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ORIGIN, COMPOSITION, AND FUNCTION OF GLYCOCONJUGATES IN THE CEPHALIC REGION IN LARVAE OF CULICIDAE AND SIMULIIDAE (DIPTERA: CULICOMORPHA)

by

Kenneth M. Fry

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

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EDMONTON, ALBERTA

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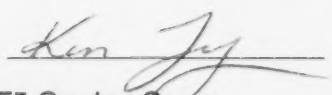

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Origin, composition, and function of glycoconjugates in the cephalic region in larvae of Culicidae and Simuliidae (Diptera: Culicomorpha)** submitted by **Kenneth McNichol Fry** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in **Entomology**.

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DEDICATION

I dedicate this thesis to my dear friend and wife, Constance Eileen Fry, for her inexhaustible encouragement, compassion, and love.

ABSTRACT

Histochemical techniques were used to determine the origin, composition, and function of glycoconjugates in the cephalic region of larval mosquitoes and black flies. Sections of late instar larvae of Aedes aegypti (L.), Aedes implicatus Vockeroth, Culex territans Walker, Anopheles earlei Vargas (Culicidae), Simulium vittatum Zetterstedt and Gymnopais dichopticoides Wood (Simuliidae) were stained with alcian blue at pH 0.5, 2.5, and 3.2, with aldehyde fuchsin, and with periodic acid-Schiff's reagent to determine the chemical affinities of glycoconjugates present in the cephalic region. Lectins derived from Triticum vulgaris (WGA) specific for N-acetyl-β-D-glucosamine, Bandeiraea simplicifolia (BS I) specific for α-D-Galactose, Sophora japonica (SJA) specific for N-acetyl-β-D-galactose, Pisum sativum (PSA) specific for α-D-mannose, and Ulex europaeus (UEA I) specific for α-L-fucose were used to determine the composition of the secretion product of the dorsal and ventral cephalic glands in larvae of Aedes aegypti (L.). Additionally, larvae were fed inert Dayglo® particles and then sectioned and stained to determine the origin of glycoconjugates reported in the gut contents. Examination showed that weakly and strongly sulphated glycoconjugates and carboxylated glycoconjugates secreted from what are described here as dorsal and ventral cephalic glands and which are associated with the labral, mandibular, and maxillary epidermis, are not used to enhance food capture or handling. Instead, it is hypothesized that these glycoconjugates aid in formation of mouthpart structures during the larval stadium, or facilitate ecdysis. Of the lectins, only PSA bound to cells in the ventral cephalic gland, indicating the presence of α-mannose. Glycoconjugates, previously observed in the gut and on the surface of the mouthparts are shown to be of exogenous origin, to be associated with particulate food and possibly to render food particles self-agglutinating. This

latter quality is important to larvae as it facilitates food acquisition. Removing fine and coarse particulate organic matter from suspension and reforming it as larger faecal pellets is an example of nutrient spiraling and resource refinement and alteration within an aquatic ecosystem.

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LIST OF ABBREVIATIONS

+	weakly positive
++	positive
+++	strongly positive
-	negative
?	unknown or unspecified
A	acidic glycoconjugate
a-Fuc	a-L-fucose
a-Gal	a-D-galactose
a-GalNAc	N-acetyl-a-D-galactosamine
a-Man	a-D-mannose
Ad	adult
Ant	antenna
APBr	anteromedian palatal brush
b	brain
b-GalNAc	N-acetyl-b-D-galactose
b-GlcNAc	N-acetyl-b-D-glucosamine
BS I	agglutinin from Bandeiraea simplicifolia
C	cuticle
Cg	carboxylated glycoconjugate
Ca	cardia
CS	chondroitin sulphate
Dcg	dorsal cephalic gland
Dp	Dayglo® particles
ep	epipharynx
f	pharyngeal fringe

fp	food particles
fs	fan stem
GLC	glycoconjugate
H	hypostome
Hy	hyaluronic acid
L	larva
LF	labral fan
LM	labral muscle
LPB	lateral palatal brush
LPBE	lateral palatal brush epidermis
LPPA	lateral palatal pennicular area
Md	mandible
ME	midgut epidermis
Mx	maxilla
Mx. brush	maxillary brush
MxBr	maxillary brush
N	neutral glycoconjugate
pb	palatal brush
per. memb.	peritrophic membrane
Pha fringe	pharyngeal fringe
Pha	pharynx
pLB	pharate labral brush
pLF	pharate labral fan
pLPB	pharate lateral palatal brush
pm	peritrophic membrane
PSA	agglutinin from Pisum sativum
Pu	pupa

S	sialic acid
scler. cut.	sclerotized cuticle
sd	salivary duct
Sg	suboesophageal ganglion
SJA	agglutinin from Sophora japonica
SS	strongly sulphated glycoconjugate
To	torma
uC	unsclerotized cuticle
UEA I	agglutinin from Ulex europaeus
unscler. cut.	unsclerotized cuticle
Vcg	ventral cephalic gland
WGA	wheat germ agglutinin or agglutinin from Triticum vulgaris
WS	weakly sulphated glycoconjugate

I. INTRODUCTION

Black flies and mosquitoes have received considerable attention from the scientific community for several reasons including their nuisance to humans, harassment of livestock resulting in reduced weight or vitality, and most importantly, their ability to serve as vectors of disease-causing organisms (Harwood & James 1979). Mosquitoes serve as a vector of the disease-causing organisms of a wide variety of diseases, including malaria, yellow fever, dengue, and several encephalitides (Harwood & James 1979). Currently, approximately 270 million cases of malaria, resulting in nearly 1 million deaths a year are estimated (Clements 1992).

Black flies are the vector for onchocerciasis, or river blindness, which has resulted in 340,000 cases of blindness per year (WHO 1987). Although under control in Africa, and a success story to be publicized (Crosskey 1990), onchocerciasis cases are increasing worldwide. As rain forest is cleared in Central and South America, immigrant populations are being exposed to the disease. Indeed, as development continues there and in Africa and Southeast Asia, many more people will be exposed to diseases vectored by arthropods, particularly black flies and mosquitoes.

With regard to disease vectors and their control, there is a movement toward using environmentally compatible methods. To effectively control a vector with little or no disturbance to the environment, as much as possible about the vector should be understood. Most research into the biology of larvae of black flies and mosquitoes, the semaphoront easiest to control, has addressed morphology and behaviour. Several recent extensive and detailed works on feeding by simuliid (Figs. I-1, I-2) and culicid larvae (Figs. I-3, I-4) (Chance 1970; Dahl et al. 1988; Dahl et al. 1990; Lacoursiére 1992;

Lacoursiére & Craig 1993; Merritt et al. 1992a) have combined analyses of morphology, behaviour, and interaction with the physical environment, in particular, hydrodynamics, to elucidate the salient factors involved in food-particle capture. Information of this nature, it is hoped, will facilitate accurate targeting with control substances, whether synthetic or natural, so as to have the least impact on non-target organisms, the least economic cost, and a more efficient effect on target species. Among the areas of research listed above, one has been the subject of considerable debate; the role of glycoconjugates in food-particle capture or handling.

Glycoconjugates are widespread within the Animalia (Cássaro & Dietrich 1977) and many forms have been reported in many invertebrate taxa (Table I-1). Analysis of filter-feeding systems by Jørgensen (1975) and Jørgensen et al. (1984) has yielded evidence of widespread use of glycoconjugates in conjunction with various organs used in procuring food particles.

Bivalves, including oysters, mussels, scallops, clams, and cockles, are the principal filter feeders in many fresh- and saltwater benthic habitats (Jørgensen 1975). Organelles implicated in food acquisition have been studied, including flagella in flagellates, paroral membranes in ciliates, choanocyte collars in choanoflagellates and sponges, cilia in bryozoans, brachiopods, phoronids and many other metazoans, and maxillae in copepods and other crustaceans (Jørgensen et al. 1984).

In addition to these mechanical means of food-particle capture, the role of glycoconjugates in feeding has been studied in invertebrates (Jørgensen 1966; Jørgensen et al. 1984). Mucus sheets or nets used in feeding have been reported for polychaetes, gastropods, bivalves, echiuroid worms, and echinoderms (Jørgensen 1966). The freshwater triclad, Bdellocephala punctata (Pallas), is aided by mucous-entanglement in the capture of some prey items

(Adams 1980). Chaetopterus variopedatus (Renier and Clapérede), a polychaete worm, secretes mucus from the posterior medial edge of aliform notopods on the 12th segment to filter food particles from water (Flood & Fiala-Médioni 1982). The food-containing mucous net is roiled into a ball and transported to the mouth for ingestion.

Within Insecta, mucus or silk nets are used by the tube-dwelling trichopterans Phylocentropus (Wallace et al. 1976), Protodipseudopsis (Gibbs 1968), Macronema and Hydropsyche (Ross 1944), and some philopotamid larvae (Wallace & Merritt 1980). Within the Nematocera, capture nets are most commonly seen in the chironomids, Chironomus plumosus L. (Walshe 1951), Endochironomus Meigen (Oliver 1971), and Rheotanytarsus Kieffer (Walshe 1951).

The potential use of glycoconjugates to aid in food-particle capture by black flies was recognized by Strickland (1911) in a study of Simulium Latreille larvae. He suggested that a "sticky" substance originating from "pharyngeal glands" might be used to clean the mouthparts during suspension feeding. More recently Ross and Craig (1980) reassessed glycoconjugate use by black fly larvae. They suggested that glycoconjugates were deposited onto the labral fans to enhance particle capture. Similarly, Merritt and Craig (1987) described the use of glycoconjugates in food acquisition by mosquito larvae. Subsequent work on black fly larvae by Lacoursiére and Craig (Lacoursiére 1992; Lacoursiére & Craig 1993) and mosquito larvae by Dahl et al. (1988) and Fry and McIver (1990) has cast doubt on the hypothesis of particle capture aided by glycoconjugates. However, LaBarbera (1984) has stated that physical properties of food-particle capture surfaces can greatly influence capture efficiencies. In the case of larval black flies and mosquitoes, glycoconjugates deposited on the labral structures could enhance food-particle capture.

The aim of this project was to determine the origin, composition, and function of glycoconjugates in the cephalic region of larval culicids and simuliids. This topic has application within the broad context of an increased awareness of deleterious effects on non-target organisms of insecticide use to control larval black flies and mosquitoes. A better understanding of what constitutes acceptable foodstuffs for culicids or simuliids versus material that is not sampled, and a thorough understanding of the means of food-particle capture of culicids and simuliids will potentially result in the refinement of insecticide formulations. Insecticides may be produced that are consumed specifically by culicids or simuliids and not by non-target organisms.

Additionally, a general curiosity toward mechanisms of suspension feeding systems, and specifically the unresolved role of glycoconjugates in food-particle capture also provide a motive for addressing this topic.

The hypothesis that glycoconjugates are produced in the cephalic region and secreted externally for use in food-particle capture, or handling, is tested for larval simuliids and culicids.

Chapter 2 describes a histochemical study of glycoconjugates originating from dorsal and ventral cephalic glands in 3rd and 4th instars of Aedes aegypti (L.), A. implicatus Vockeroth, Anopheles earlei Vargas, and Culex territans Walker. This assemblage of species includes representatives from the major behavioural groups within the functional feeding group "collector-filterer" (Merritt & Cummins 1984; Merritt et al. 1992b) in North American mosquitoes; namely surface feeding An. earlei, water column feeding C. territans, and bottom feeding A. aegypti and A. implicatus. Specimens of A. aegypti were acquired from a laboratory colony of long standing in the Department of Entomology, University of Alberta. To compensate for any behavioural artifacts associated with a colony population, specimens of another bottom feeding species, A.

implicatus, were collected from the field.

Chapter 3 is a histochemical evaluation of glycoconjugates originating from dorsal and ventral cephalic glands in late instar larvae of Simulium vittatum Zetterstedt and Gymnopais dichopticoides Wood. S. vittatum is a locally available species that has well developed labral fans for filter feeding. G. dichopticoides larvae are fanless and graze on the substrate for food. The inclusion of G. dichopticoides in the study was to test whether glycoconjugates are used in food acquisition (specifically filtering), food handling (filtering or grazing), or both.

Chapter 4 is a cytochemical analysis using lectins of glycoconjugates in the cephalic region of 3rd instar A. aegypti larvae. This analysis is an initial step towards establishing the role of glycoconjugates originating from the dorsal and ventral cephalic glands and filling apolysial spaces. This chapter includes a discussion of the falsification of the original hypothesis regarding the function of glycoconjugates in the cephalic region. Additionally, an alternative hypothesis to account for the presence of glycoconjugates in the cephalic region is presented.

The 5th and final chapter is a summary of the results and conclusions arising from this project and includes suggestions for further research. Following the conclusion are two appendices. Appendix I lists the formulae for chemical solutions used in the histo- and cytochemical evaluation of glycoconjugates. Appendix II lists the staining methods used in evaluating glycoconjugates.

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TABLE I-1. A SURVEY OF STRUCTURAL, SECRETORY, OR FEEDING-ASSOCIATED GLYCOCONJUGATES IDENTIFIED IN INVERTEBRATA

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
MOLLUSCA					
GASTROPODA					
PROSOBRANCHIA					
Rissoa parva	Ad	A	mantle	?	Gostan 1960
Pila virens	Ad	S, SS, Hy, Cg	mantle	shell calcification (dorsal epidermis),protection (ventral epidermis)	Devi et al. 1983
Busycon spp.		CS		fibre binding	Hunt 1970
Buccinum undatum	Ad	A	hypobranchial gland	cleanse mantle cavity	Hunt 1973
Lymanaea stagnalis	Ad	N, SS, WS	epidermal & subepidermal gland cells of mantle & foot	lubrication of foot	Zylstra 1972
Biomphalaria pfeifferi	Ad	N, SS, WS	epidermal & subepidermal gland cells of mantle & foot	lubrication of foot	Zylstra 1972
PULMONATA					
Helix aspersa	Ad	A	mantle	?	Campion 1961
Arion rufus	Ad	N	mantle	?	Binot & Chetail 1968
Lehmania poireiri	Ad	A	mantle	?	Arcadi 1963, 1967
BIVALVIA					
PTERIOMORPHIA					
Barbatia obliquata	Ad	SS	epithelial & subepithelial glands of mantle	calcification of shell	Rupavathi et al. 1984

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
Barbatia obliquata	Ad	Cg	subepithelial glands of mantle	calcification of shell	Rupavathi et al. 1984
HETERODONTA					
Mercenaria mercenaria	Ad	SS	mantle	calcification of shell	Hillman 1968
ARTHROPODA					
CHELICERATA					
MEROSTOMATA					
XIPHOSURA					
Limulus spp.	Ad	CS		fibre binding	Hunt 1970
UNIRAMIA					
INSECTA					
ZYGENTOMA					
Ctenolepisma longicauda	Ad	A	cuticle, midgut epithelium	?	Day 1949
HEMIPTERA					
Reduviidae					
Rhodnius prolixus	L	Hy	dermal glands	facillitate shedding of exuvia	Baldwin & Salthouse 1959
Gnorimoschema operculella	L	A	cuticle	?	Day 1949
Lygaeidae					
Oncopeltus fasciatus	Ad	N	maxillary glands	?	Linder 1956
Oncopeltus fasciatus	Ad	A	mandibular glands	?	Linder 1956
Oncopeltus fasciatus	Ad	N, A	salivary glands	forms stylet sheath	Salkeld 1960
Cercopidae					
Aeneolamia var. saccharina	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a,b
Philaenus leucophthalmus spumarius	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a,b
Clovia lineatocollis	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a,b

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
Cosmoscarta abdominalis	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a
Neophilaenus campestris	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a
Chaetophyes compacta	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a
Chaetophyes compacta	L	A	malphigian tubules	form larval tube	Marshall 1968
Machaerota coronata	Ad	SS	malphigian tubules	stabilize spittle bubbles	Marshall 1966a
Machaerota coronata	L	A	malphigian tubules	form larval tube	Marshall 1968
Pectinariophytes stalii	L	A	malphigian tubules	form larval tube	Marshall 1968
Aspongopus janus	Ad	SS	salivary glands	?	Verma & Sinha 1980
Laccotrephes maculatus	Ad	Cg, WS	salivary glands	?	Verma & Sinha 1980
DICTUOPTERA					
Blatellidae					
Periplaneta americana	Ad	A	foregut, peritrophic memb., salivary glands	?	Day 1949
Periplaneta americana	Ad	SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Periplaneta americana	Ad	Hy, SS, CS	area surrounding central mass of nerve fibres of meso- & metathoracic ganglia	?	Ashurst 1961, 1984; Ashurst & Costin 1971a
ORTHOPTERA					
Acrididae					
Locusta migratoria	Ad	A	foregut, caeca, midgut, salivary glands	?	Day 1949

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
Locusta migratoria	Ad	N, SS	connective tissue surrounding ejaculatory duct	?	Ashurst & Costin 1971a
Locusta migratoria	L	Hy	glial lacunar system	?	Ashurst 1984
Locusta migratoria	Ad	Hy, SS	thoracic ganglia glial lacunar system & neural lamella	?	Ashurst & Costin 1971b
Acrida turrita	Ad	S, CS, N, SS, A	male accessory gland	?	Verma et al. 1981
Oxya velox	Ad	S, N	male accessory gland	?	Verma et al. 1980
PHASMIDA					
Phasmatidae					
Carausius morosus	Ad	CS, Hy	thoracic ganglia neural lamella, glial lacunar system	?	Ashurst & Costin 1971c
COLEOPTERA					
Dytiscidae					
Graphoderus occidentalis	Ad	A	labial palp glands	facilitate movement in water, prevent desiccation, essential to sensory structures	Leung & Zacharuk 1987
Tenebrionidae					
Tenebrio molitor	Ad	A	midgut, malphigian tubules	?	Day 1949
Tenebrio molitor	L	A	midgut, fatbody	?	Day 1949

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
Curculionidae					
Odontopus nigricornis	Ad	Cg, SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
LEPIDOPTERA					
Pieridae					
Pieris rapae	L	A	midgut	?	Day 1949
Pyralidae					
Anagasta kuhniella	Ad	A	cuticle, peritrophic memb.	?	Day 1949
Galleria mellonella	Ad	A	extracts of midgut	?	Estes & Faust 1964
Galleria mellonella	Pu	CS, N	neural lamella & dorsal connective of CNS	?	Ashurst & Costin 1971c
Tineidae					
Tineola bissenlliella	L	A	midgut, fat body	?	Day 1949
Saturniidae					
Attacus ricini	L	Hy	peritrophic memb.	?	Kawakita 1961
Samia cynthia	L	Hy	peritrophic memb.	?	Kawakita 1961
Theophila mandarina	L	Hy, CS	peritrophic memb.	?	Kawakita 1961
Bombycidae					
Bombyx mori	Ad	A, Hy	peritrophic memb.	?	Nisizawa et. al. 1963; Kawakita 1961
Bombyx mori	L	Hy	peritrophic memb.	?	Kawakita 1961
Hemerophilidae					
Hemerophila artilinara	L	Hy, CS	peritrophic memb.	?	Kawakita 1961
Euproctis scintillans	L	SS, N	salivary glands	?	Verma & Sinha 1980

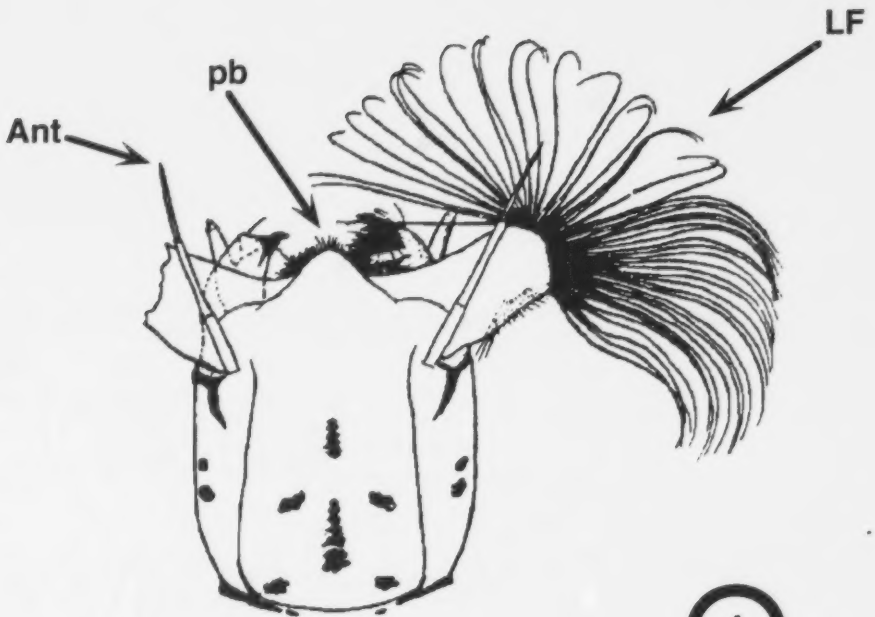
TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
HYMENOPTERA					
Apidae					
Apis mellifera	Ad	A	crypt cells of midgut, salivary glands	?	Day 1949
Apis mellifera	Ad	N, A, Hy	peritrophic memb.	?	Pabst et al. 1988
Apis indica	Ad	SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Apis indica	Ad	Hy, Cg, WS, SS, N, S	salivary glands	protection during digestion	Vadgama & Kamat 1973a
Ichneumonidae					
Pimpla turionellae	Ad	Hy	uterus gland	alter ionic milieu in host	Osman & Fuhrer 1979
Pimpla turionellae	Ad	N	poison gland	prevent egg encapsulation	Osman & Fuhrer 1979
Anthophoridae					
Xylocopa pubescens		Cg, Hy	comb cells	coat inside cells	Gerling et al. 1979
DIPTERA					
Culicidae					
Anopheles quadrimaculatus	L	A	labral glands	aid food acquisition	Merritt & Craig 1987
Culex pipiens fatigans	Ad	A	accessory glands	?	Adlakha et al. 1976
Culex pipiens pipiens	L	A	pharynx, anterior midgut	?	Dahl et al. 1990
Culex pipiens	L	SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Culex torrentium	L	A	pharynx, anterior midgut	?	Dahl et al. 1990

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
Culiseta morsitans	L	A	pharynx, anterior midgut	?	Dahl et al. 1990
Aedes communis	L	A	pharynx, anterior midgut	?	Dahl et al. 1990
Aedes triseriatus	L	A	labral glands	aid food acquisition	Merritt & Craig 1987
Simuliidae					
Prosimulium sp.	L	A	dorsal & ventral glands	aid food acquisition	Ross & Craig 1980
Stegopterna sp.	L	A	dorsal & ventral glands	aid food acquisition	Ross & Craig 1980
Cnephia sp.	L	A	dorsal & ventral glands	aid food acquisition	Ross & Craig 1980
Simulium sp.	L	A	dorsal & ventral glands	aid food acquisition	Ross & Craig 1980
Chironomidae					
Chironomus tentans	L	CS, SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Chironomus plumosus	L	Hy, SS	salivary glands	cement for secretions	Defretin 1951
Smittia spp.	L	SS, Hy, N	salivary glands	?	Kato & Sirlin 1963
Sciaridae					
Bradysia mycorum	L	SS, Hy, N	salivary glands	?	Kato & Sirlin 1963
Muscidae					
Musca domestica nebulo	L	A	brain connective tissue, ganglia, imaginal disks	?	Mustafa & Kamat 1970
Musca domestica	L	A	salivary glands	?	Day 1949
Musca domestica	L	A	thoracico-abdominal ganglion neural lamella	protective covering	Sharma 1983

TAXON	STAGE	GLC	LOCATION	SUGGESTED FUNCTION	REFERENCE
Glossina morsitans morsitans	Ad	N	accesory gland	component of spermatophore wall	Odhiambo et al. 1983
Calliphoridae					
Calliphora vicina	Ad	A, Hy?	outer receptor lymph space of haltere campaniform sensilla	protection of dendrite from desiccation, regulation of ionic composition of receptor lymph, protection from mechanical stimuli	Grunert & Gnatzy 1987
Calliphora erythrocephala	Ad	Hy	olfactory sensilla	?	Pietra et al. 1980
Calliphora erythrocephala	Ad	Hy, CS	whole body extract	?	Mustapha & Kamat 1970
Phormia regina	L	S S	whole body extracts	?	Sharief et al. 1973
Phormia regina	Ad	A	trichogen & tormogen cells of labellar hairs, wing hairs	barrier to modulate sensory response	Pietra et al. 1980
Lucilia cuprina	L	A	cuticle, fat body, peritrophic memb., salivary glands	?	Day 1949
Lucilia cuprina	Ad	A	foregut	?	Day 1949
Chrysomyia rufifacies	L	SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Sarcophagidae					
Sarcophaga bullata	L	Cg, SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Drosophilidae					
Drosophila melanogaster	L	SS, S	salivary glands	?	Vadgama & Kamat 1969, 1973a, b
Drosophila auraria	L	S S	salivary glands	?	Thomopoulos et al. 1989

Fig. I-1. Dorsal aspect of a larval black fly head (after Crosskey 1960).

Fig. I-2. Longitudinal section through a larval black fly head. Scale bar = 500 µm (after Grenier 1949).



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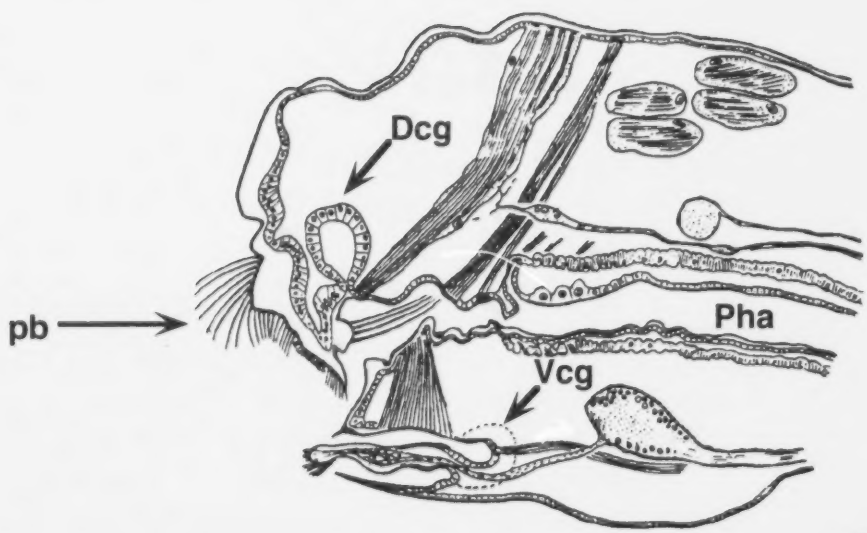
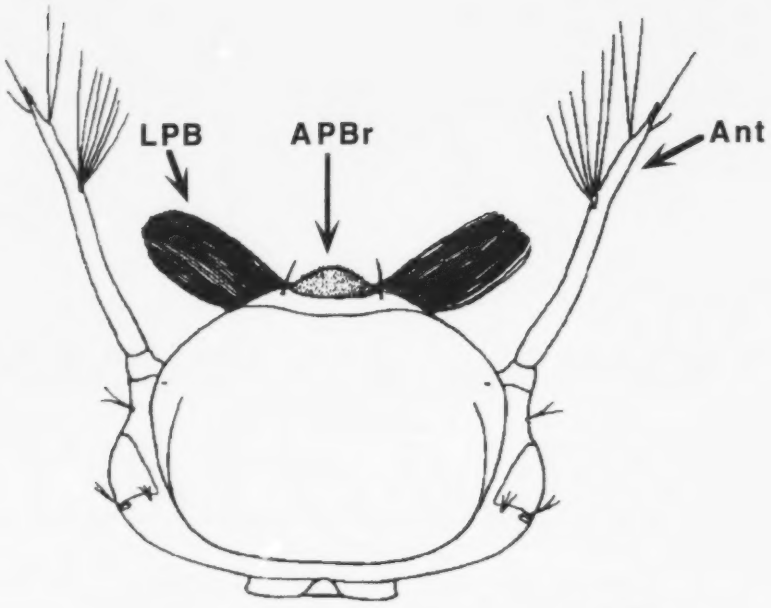


2

Fig. I-3. Dorsal aspect of a larval mosquito head (after Merritt & Cummins 1984).

Fig. I-4. Schematic illustration of a larval mosquito head. Scale bar = 500 μm (after Dahl et al. 1988).



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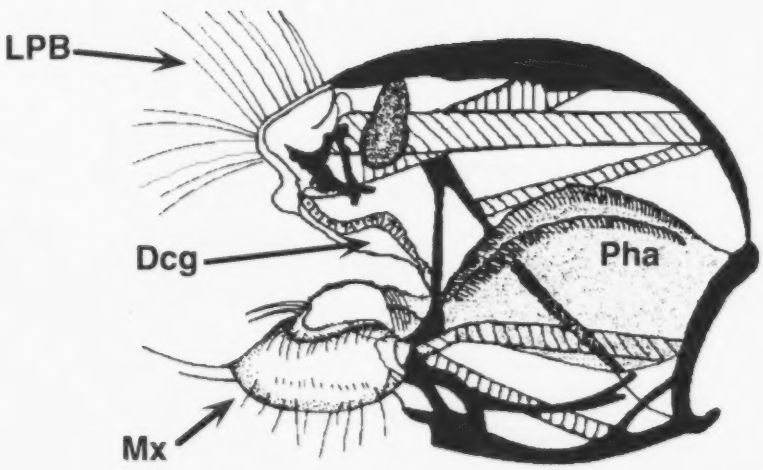

4

II. ORIGIN AND FUNCTION OF GLYCOCONJUGATES ASSOCIATED WITH FEEDING IN MOSQUITO LARVAE (DIPTERA: CULICIDAE)

II.1 SYNOPSIS

Histochemical techniques were used to determine the origin and function of glycoconjugates in the cephalic region of larval mosquitoes. Sections of late instars of Aedes aegypti (L.), Aedes implicatus Vockeroth, Culex territans Walker, and Anopheles earlei Vargas were stained with alcian blue at pH 0.5, 2.5, and 3.2, with aldehyde fuchsin, and with Schiff's reagent to determine the chemical affinities of glycoconjugates present in the cephalic region. Additionally, larvae were fed inert Dayglo® particles and then sectioned and stained as above to determine the origin of glycoconjugates reported in the gut contents. Examination showed that sulphated and carboxylated glycoconjugates secreted from what are described here as dorsal and ventral cephalic glands and which are associated with the labral, mandibular, and maxillary epidermis, are not used to enhance food capture or handling. Instead, it is hypothesized that these glycoconjugates aid in formation of mouthpart structures during the pharate stage, or facilitate ecdysis. Glycoconjugates, previously observed in the gut and on the surface of the mouthparts, are shown to be of exogenous origin, and associated with particulate food. They may render food particles self-agglutinating which is probably important to larvae in securing food by removing fine and coarse particulate organic matter from suspension and in reforming it as larger faecal pellets.

II.2 INTRODUCTION

The feeding behaviour of mosquito larvae has been described as "collecting-filtering" by Merritt et al. (1992a) and as "non-predatory and omnivorous" by Aly (1985). To better understand filter feeding in culicid larvae, Harbach (1977), Pucat (1965), Shalaby (1957a,b,c,d), and others used a variety of approaches. The morphological and behavioural studies suggested sieving by the lateral palatal brushes as the principal mechanism of food acquisition.

As part of a comprehensive study of filter feeding in larval culicines, Dahl et al. (1988) used a systems approach to identify important spatial and temporal elements. They separated feeding into two discrete phases. During the first phase contraction of median labral retractor muscles cause the tormae to turn on their axes, transmitting force to elastic lateral palatal plates, which, in turn, cause the lateral palatal brushes to adduct into the buccal cavity. This results in water with entrained food particles flowing towards the buccal cavity. The second phase occurs when the water is purportedly sucked into the expanding pharynx and filtered by pharyngeal fringes. The lateral palatal brushes are then abducted by release of elastic tension in the lateral palatal plates caused by relaxation of the labral retractor muscles. Water is then expelled by a posterior to anterior wave of contraction in the pharynx.

Widahl (1992) addressed the problem of particle acquisition on a broader scale. Flow patterns near individual larvae were analyzed using high-speed video. Two large flows, β and γ, directed toward the buccal cavity were identified. A third flow, α, is partially recirculated into the β and γ flows, and, also, creates a toroidal vortex anterior to the larva. Widahl (1992) did not know if the α flow resulted solely from contraction of the pharynx, or by the pharynx and mouthparts acting together.

Both the above models for filter feeding in culicines imply that the pharynx plays a significant role in particle capture (Dahl et al. 1988). This is in contrast to previous models proposing the lateral palatal brushes or the mandibles and maxillae as the primary-filtering organs (Surtees 1959; Christophers 1960; Merritt & Craig 1987).

In anopheline larvae, the lateral palatal brushes act as paddles to direct a flow of water towards the buccal cavity. But, contrary to the mechanism proposed in culicines (Dahl et al. 1988), Merritt and coworkers hypothesized that mandibular-sweeper setae and maxillary-brush setae direct food into the pharynx where the pharyngeal fringes clean food off of the mandibular-sweeper setae (Merritt et al. 1992b). Schremmer (1949) reported that anophelines used the maxillae to direct food into the pharynx where it was filtered. However, the precise mechanism for particle capture in culicine and anopheline larvae remains undetermined (Clements 1992; Merritt et al. 1992a).

The role of mucus in particle capture by invertebrates is well documented (Jørgensen 1966, 1983; Jørgensen et al. 1984). However, its role in larval culicid and simuliid feeding is still subject to debate. Ross and Craig (1980), using histochemical techniques, identified secretory mucous glands in simuliid larvae and discussed the role of mucus in food acquisition. Merritt and Craig (1987), in a study of larvae of Aedes triseriatus (Say) and Anopheles quadrimaculatus Say, hypothesized that lateral palatal brush epidermal cells produced mucus that was subsequently deposited onto the surface of the lateral palatal brush filaments to enhance food-particle capture. This hypothesis was reinforced by the interpretation by Craig (1974), Davies (1974), and Wood (1978) that labral fans in simuliids and lateral palatal brushes in culicids are serially homologous.

Contrary to the assertion of Merritt and Craig (1987), Dahl et al. (1988) did not accept the presence or use of mucus in filter feeding by culicid larvae. Futhermore, Dahl et al. (1990) reported that mucus, although present in the foregut of culicid larvae, was neither in nor on the lateral palatal brushes, nor were secretory glands observed in the material studied. Additionally, Fry and McIver (1990) showed that, in larvae of Aedes aegypti (L.), the lateral palatal brush epidermis did not secrete mucus onto the surface of the lateral palatal brush filaments. Instead, these cells had the major function of producing the filaments of the large lateral palatal brush of the ensuing larval instar (Fry & McIver 1990). They showed also the near-complete degeneration of this epidermis in the fourth instar, supporting the hypothesis of a primarily developmental role for this epidermis.

The processes resulting in formation of lateral palatal brushes in culicids and of labral fans in simuliids is extraordinary (Craig 1974; Fry & McIver 1990). Immediately following ecdysis, in all larval instars except the last, the epidermis underlying labral structures apolyses from the larval cuticle to allow for formation of the labral structures of the next instar. In culicid larvae, a large apolysial space develops between the labral cuticle and the apex of the epidermal cells. This space, filled with material of unknown origin and function (Fry & McIver 1990), is continuous with that between head capsule cuticle and epidermis throughout the head.

Unlike head capsule cuticle, where the procuticle is broken down and reabsorbed for recycling, the procuticle of the labral structures is not broken down and reabsorbed (Fry & McIver 1990). The process of apolysis, secretion of moulting gel, ecdysial membrane formation, procuticle digestion and reabsorbtion, and subsequent secretion of new cuticle does not occur for labral structures. Instead, apolysis of the lateral palatal brush epidermis yields a large

fluid-filled space allowing for development of the new lateral palatal brush. No digestion of procuticle occurs and it is doubtful that the fluid filling the apolysial space serves the same purpose as moulting gel does elsewhere in the larva.

With the putative role and presence of mucus in particle capture so uncertain, the goal of this project was to histochemically determine the origin and function of material in the cephalic region of larval mosquitoes. The term "mucus" implies a function as a viscous material for particle capture (Jørgensen et al. 1984), or for lubrication (Clamp et al. 1978; Denny & Gosline 1980). Because the function of the so-called mucus in mosquito larvae is so uncertain, the chemically and functionally neutral term "glycoconjugate" (Reid & Clamp 1978) is used instead.

The terms mucus, mucosubstance, mucopolysaccharide, mucin, and glycosaminoglycan refer to a substance that is comprised mainly of sugar residues, in particular hexosamine-containing polysaccharides (Cook 1982). Spicer et al. (1965) have argued that the terms mucin or mucopolysaccharide be reserved for those substances that are of human connective tissue origin. They also state that carbohydrates isolated from any tissue should be labeled according to histochemical and biochemical properties. The term glycoconjugate simply signifies that sugar residues are covalently bound to other sugars, proteins, or lipids. Until specific a substances' biochemical properties are determined, it would be presumtuous to identify it as being a glycoprotein, glycolipid, glycosaminoglycan or polysaccharide.

II.3 MATERIALS AND METHODS

Third and fourth instar Culex territans Walker (n=22) and third instar Anopheles earlei Vargas (n=16) were collected from a semi-permanent slough west of Edmonton, Alberta during the summer of 1990. Third instar Aedes implicatus Vockeroth (n=51) larvae were collected from a roadside ditch south of Edmonton in the spring of 1989. Third instar Aedes aegypti (L.) (n=130) larvae were obtained from a laboratory colony maintained in the Department of Entomology, University of Alberta. Larvae were fixed in alcoholic Bouin's for 24 hours, dehydrated in a graded ethanol series, embedded in TissuePrep® paraffin wax (melting point 56-57°C) (Fisher Scientific Co.), and sectioned at 10 µm on a Reichert-Jung rotary microtome. Serial sections were stained with alcian blue at pH 1.0, 2.5, and 3.2 (Bancroft and Stevens 1990), with alcian blue pH 2.5-aldehyde fuchsin (Spicer et al. 1962), and with alcian blue pH 2.5-periodic acid-Schiff's reagent (Mowry 1963)(Table II-1). Alcian blue electrostatically binds to polyanions bearing sulphate or carboxyl groups depending upon the ionization state of the parent sugar residues (Bancroft & Stevens 1990), and is widely accepted as an indicator of glycoconjugates (Cook 1982). Schiff's reagent, which binds to neutral glycoconjugates, and aldehyde fuchsin, which binds to sulphated glycoconjugates, complement alcian blue staining by competing for binding sites at different ionizing pH levels.

Control specimens consisted of 10 µm sections of alcoholic Bouin's-fixed, paraffin-embedded, human colon biopsy material and 5 µm sections of Bouin's-fixed, paraffin embedded, Trichoplusia ni (Hübner) larvae (Lepidoptera: Noctuidae). Mucus in the goblet cells of the human colon samples provided a positive control for alcian blue staining (Allen 1978). The noctuid specimens,

fed a known diet (B.A. Keddie, personal communication), provided invertebrate material for comparison of gut content and peritrophic membrane staining reaction.

A test to determine the origin of glycoconjugates in mosquito larval guts (Dahl et al. 1990) was conducted using Dayglo® particles (Dayglo Color Corporation, Cleveland, Ohio). Dayglo® particles are chemically inert to alcian blue, Schiff's reagent and to aldehyde fuchsin (unpublished results). Fifty 3rd instar A. aegypti, reared in tap water containing algae, bacteria, fine and coarse particulate organic matter, and diatoms, were transferred to a 2 L tank containing distilled water and a suspension of 0.5 g Dayglo® particles. To serve as a feeding stimulant (Dadd 1970; Aly 1983), 0.5 g of brewer's yeast was suspended in 500 ml distilled water, heated to 60°C, allowed to cool to room temperature, and filtered through a 0.22 µm millipore syringe filter. Twenty-five ml of filtrate were added to the Dayglo® tank. The larvae were allowed to feed on Dayglo® particles for 20 minutes and were then processed and stained as above (Table II-1). Any staining observed in the region of the gut containing Dayglo® particles would have had to originate from the larva and not from an exogenous source. Alternatively, if no staining was observed in the region of the gut containing Dayglo® particles, then staining in the region without Dayglo® particles must be due to material of exogenous origin.

Serial sections of all material were examined using a Reichert-Jung Polyvar photomicroscope and recorded on Kodak Ektachrome T160 colour film.

Morphological terms follow those of Harbach and Knight (1980).

II.4 RESULTS

The results of staining were similar for each species of mosquito examined unless otherwise noted (Table II-2).

Larvae stained with alcian blue showed uniformly weak alcianophilia at pH 0.5 (Fig. II-1) and 3.2 (Fig. II-2) and strong alcianophilia at pH 2.5 (Fig. II-3) in newly-deposited, unsclerotized cuticle throughout the head capsule and mouthparts (pLPB). Unsclerotized (Fig. II-4 pLPB) and newly-forming cuticle (Fig. II-4 arrowhead) was Schiff's reagent (PAS) positive. Apodemes and tracheal cuticle exhibited weak to strong alcianophilia (Figs. II-1–II-3). Wherever muscle inserted (To), broad bands of material between the muscle and insertion point were weakly alcianophilic at pH 0.5 and 3.2 and strongly alcianophilic at pH 2.5 (Figs. II-1, II-4). The apolysial space enclosed by the lateral palatal brush epidermis posteriorly, laterally and dorsally, the lateral palatal pennicular area anteriorly, and epidermis ventrally, contained flocculent material exhibiting weak alcianophilia at pH 0.5 and 3.2 and strong alcianophilia at pH 2.5 (Figs. II-1–II-3). Similar material lying in spaces created by apolysis in the mandibles, maxillae, labiohypopharynx, pharynx, and epipharynx were variously alcianophilic. Aldehyde fuchsin staining was evident as a dark purple to blue color in areas where alcianophilia was observed (Fig. II-5).

The peritrophic membrane (pm) was weakly alcianophilic at pH 0.5 and 3.2 and positively alcianophilic at pH 2.5 (Table II-2, Fig. II-5). The alcianophilia of the peritrophic membrane was uniform from point of secretion at the cardia to faecal pellet formation in the rectum. The neuropile of the brain (b), subesophageal ganglion (Sg), thoracic ganglia, and the ventral nerve cord

were weakly alcianophilic at pH 0.5 and 3.2 and mildly alcianophilic at pH 2.5 (Figs. II-1, II-2, II-5).

Paired invaginations of epidermis lateral and posterior to the dorsomentum are here recognized as ventral cephalic glands (Fig. II-1, Vcg). The lumina of the ventral cephalic glands are continuous with apolysial space resulting from invagination or apolysis of epidermis lining the dorsomentum, labiohypopharynx, mandibles and maxillae. Flocculent material in the lumina of the glands was weakly alcianophilic at pH 0.5 and 3.2 and strongly alcianophilic at pH 2.5 (Figs. II-1, II-2). There was no difference between the reaction of material in the gland lumen from that between the cuticle and epidermis of the mouthparts for each pH level used. When stained with PAS, areas of alcianophilia associated with the ventral cephalic glands were slightly purple, indicating the presence of neutral glycoconjugates.

Small paired invaginations of epidermis, ventro-medial to the lateral palatal brush epidermis, are here recognized as the dorsal cephalic glands (Dcg) (Fig. II-1). Flocculent material observed in the lumina of the dorsal cephalic glands was weakly alcianophilic at pH 0.5 and 3.2 and strongly alcianophilic at pH 2.5 (Figs. II-1, II-6). When stained with PAS, areas of alcianophilia associated with the dorsal cephalic glands were slightly purple, indicating the presence of neutral glycoconjugates. The lumina of the dorsal cephalic glands are continuous with the apolysial spaces resulting from apolysis of epidermis of the lateral palatal and antero-median palatal brushes.

Gut contents exhibited variable alcianophilia. Material recognizable as filamentous algae varied from weakly to strongly alcianophilic at all pH levels. Unidentifiable flocculent material (fp) also exhibited variable alcianophilia (Fig. II-7). Silicious diatom tests remained unstained. There was significant staining of gut content material by aldehyde fuchsin. In particular, the outer coating or

layer of some algae and unidentified material stained uniformly purple with aldehyde fuchsin. A varying reaction to PAS was seen in the gut contents.

Staining results were similar for third instars of Aedes aegypti, Aedes implicatus, Culex territans, and Anopheles earlei. Fourth instar Culex territans lacked distinct dorsal and ventral cephalic glands (Fig. II-8). Functional mouthparts are lacking in the pupal stage so no corresponding structures are required to replace the larval mouthparts. As a result, the enlarged epidermal areas posterior to the fourth instar mouthparts regress to a level appropriate for producing pupal structures. Fourth instars of the other species were not studied. However, a similar regression of lateral palatal brush epidermis was observed in fourth instar Aedes aegypti by Fry & McIver (1990).

Dayglo® particles (Dp) in the fore-, mid-, and hindgut did not stain with alcian blue, aldehyde fuchsin, or Schiff's reagent (Fig. II-7). There was no staining of material between the particles evident. Material in the gut posterior to Dayglo® particles stained variably with alcian blue, aldehyde fuchsin, or Schiff's reagent (Fig. II-7, fp), not unlike non-Dayglo®-fed larval gut contents. All other structures reacted similarly to the non-Dayglo® fed larvae.

Goblet cells in the human colon specimens reacted positively to alcian blue at pH 0.5, 2.5, and 3.2. Some gut contents and the entire peritrophic membrane of T. ni reacted positively to alcian blue in a manner similar to that of the mosquito species tested.

Close examination by bright-field and by Nomarski interference contrast microscopy at x1250 of cuticular areas adjacent to the dorsal and ventral cephalic glands failed to reveal any recognizable ductule or pore system exiting into the buccal cavity.

II.5 Discussion

Alcian blue at varying pH, aldehyde fuchsin, and periodic acid-Schiff's reagent (PAS), were used to investigate the chemical nature of glycoconjugates present in the cephalic region of mosquito larvae. Alcianophilia was observed everywhere new cuticle was forming in the head (Figs. II-1–II-3). PAS positive reactions in areas of cuticle deposition indicates the presence of neutral glycoconjugates (Fig. II-4). Based on the reactions to alcian blue at varying pH levels, it appears that most glycoconjugates in the apolysial spaces formed between cuticle and apolysed epidermis are weakly-sulphated, with a small proportion being strongly-sulphated or carboxylated (Table II-2).

A significant amount of material in the lumina of the dorsal and ventral cephalic glands exhibited strong alcianophilia. This material routinely stained more intensely than material in spaces resulting from apolysis (Fig. II-6). Glycoconjugates in cytoplasmic vesicles may be superconcentrated and become hydrated only upon discharge (Allen 1983). This might explain the difference in staining intensity between the dorsal and ventral cephalic gland lumina and the apolysial spaces. Since lumina of the cephalic glands are continuous with apolysial spaces, material found in these spaces may arise from the dorsal and ventral cephalic glands. The absence of a pore or ductule system to deliver glycoconjugates to the buccal cavity or pharynx, and the lack of staining in the gut of the region containing Dayglo® particles indicates that secretions arising from the dorsal and ventral glands are not used to aid particle capture, or handling of particles by the mouthparts.

Dahl et al. (1990) reported that material in the foregut of mosquito larvae exhibited alcianophilia. This observation is supported by staining observed in the non-Dayglo® fed larvae used in the current study. They made no mention of

alcianophilia of the peritrophic membrane. Dorner and Peters (1988) observed N-acetylglucosamine and N-acetylgalactosamine in peritrophic membranes of larvae of Aedes aegypti, Anopheles stephansi Liston and Culex pipiens fatigans Wiedemann. These sugars are also common constituents of glycoconjugates often described as mucus (Allen 1983). Numerous other insects exhibit alcianophilia of the peritrophic membrane (Table I-1). In the present study, the peritrophic membrane exhibited alcianophilia in all larvae examined (Fig. II-5).

Dahl et al. (1988) observed elongate strings of food being discharged from culicine larvae overfed with Dayglo® particles. They suggested that the particles were held together by mucus, but did not indicate the origin of the mucus. It is possible the particles were held together by glycoconjugates of exogenous origin (Ward et al. 1990). Perhaps the particles, compressed during processing by the larvae, were incorporated into a food string held together by dissolved organic matter filtered from the water column. Dissolved organic matter is presently garnering considerable attention from biologists as it relates to feeding systems in invertebrate communities (Wotton 1990a, b). What role 'exopolymers' (a term used to describe flocculent material dissolved in the water column, that may or may not arise from living organisms [Decho & Moriarty 1990]), may play in agglutination of particulate organic matter is still largely unknown.

Flocculent dissolved organic matter and glycoconjugates, or exopolymers associated with microorganisms, may be a source of glycoconjugates responsible for agglutination or aggregation of both coarse and fine particulate organic matter (CPOM and FPOM, respectively) (Merritt et al. 1992a; Ward et al. 1990). Some CPOM and FPOM, uncolonized by microorganisms, may still be sticky due to labile sugars coating particle surfaces

(Wotton 1990b). This self-agglutinating character of ingested food may account for food-bolus formation in the pharynx of mosquito larvae. It follows that the observed alcianophilia of glycoconjugates in the gut is now highly likely due to glycoconjugates associated with food items (Fig. II-7). However, several insects produce glycoconjugates in the salivary glands, presumably for use in feeding, for example, Periplaneta americana (L.), Locusta migratoria (L.), Apis mellifera L., Musca domestica L., and Lucilia cuprina Wied. (Day 1949), Oncopeltus fasciatus (Dallas) (Salkeld 1960), P. americana, Odontopus nigicornis, Apis indica, Culex pipiens pipiens, Chironomus lentanus, Chrysomyia ruffifacies, Sarcophaga bullata, and Drosophila melanogaster (Vadgama & Kamat 1969, 1973a, 1973b), Aspongopus janus, Laccotrephes maculatus, and Euproctis scintillans (Verma & Sinha 1980). A lack of alcianophilia in the region of gut containing Dayglo® particles supports the hypothesis that no glycoconjugates are produced endogenously for use in food capture or handling. In addition to the enveloping, sleeve-like, peritrophic membrane, faecal material passed out in pellets remain intact perhaps because of glycoconjugates originating from food material not digested by the larva. Inefficient absorption of nutrients and rapid transport of gut contents may also contribute to integrity of faeces (Clements 1992; Wotton 1988).

A change in food-particle size occurs during feeding by mosquito larvae. FPOM and CPOM are converted into larger faecal pellets which may be more suitable for grazers (Ward et al. 1990). This phenomenon highlights the mechanism of 'nutrient spiraling' (Webster & Patten 1979) that may involve mosquito larvae contributing material to a system in a form more attractive, or available, to other organisms. Additionally, a food resource such as FPOM or CPOM is repositioned in the habitat by removal from suspension and deposition as larger faecal pellets. Merritt et al. (1992b) reported that Anopheles

quadrimaculatus removed particulate food from the surface layers and redirected it downward in a lamellate plume of water. Alternatively, bottom-feeding aedine larvae may dislodge biofilm or exopolymeric material and resuspend it in the water column for collector-filterers (Wotton 1990a). In confined or restricted habitats, the repositioning of food resources could have significant impact on intraspecific competition (Aly 1988).

With no apparent role in food capture or handling, an alternative hypothesis is required to explain the occurrence of glycoconjugates in the cephalic apolysial space of mosquito larvae. The glycoconjugates may function in the moulting process. When deposited in the anterior labral apolysial space, they may act as a lubricant to facilitate ecdysis. In larvae of Rhodnius prolixus Stål, abdominal dermal glands are known to secrete a glycoconjugate to aid in ecdysis (Baldwin & Salthouse 1959). Alternatively, as the pharate lateral and anteromedian palatal brush and the various brushes and setae of the mandibles and maxillae are formed, glycoconjugates may act to support these structures as they develop in the apolysial space. In particular, the lateral palatal brush is relatively long and develops inside the head capsule but outside the epidermis and requires support during each larval stadium (Fry & McIver 1990).The apolysial space appears to be filled and it seems unlikely to be an external fluid or haemolymph. The dorsal cephalic glands may be the source of glycoconjugates which provide the support.

The results of this study show that glycoconjugates do not function directly in food-particle capture during filter-feeding by larval mosquitoes. However, the role of glycoconjugates in larval development requires further study. The precise composition of glycoconjugates secreted by the dorsal and ventral cephalic glands remains unknown. The use of precise cytochemical

techniques to determine the composition of the glycoconjugates will facilitate understanding of their role in larval mosquito development.

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TABLE II-1. APPORTIONMENT OF MOSQUITO LARVAE STAINED WITH ALCIAN BLUE, ALDEHYDE FUCHSIN, AND PERIODIC ACID-SCHIFF'S REAGENT

Species	Stain				
	Alcian Blue			Aldehyde	Periodic
	pH 1.0	pH 2.5	pH 3.2	Fuchsin	Acid-Schiff's Reagent
Aedes aegypti	10	22	13	12	13
Aedes implicatus	21	11	12	8	9
Anopheles earlei	3	5	3	3	2
Culex territans	4	6	4	4	4

TABLE II-2. GLYCOCONJUGATE STAINING IN LARVAE OF AEDES AEGYPTI, AEDES IMPLICATUS, ANOPHELES EARLEI, AND CULEX TERRITANS

Structure	Alcian Blue			Aldehyde Fuchsin	Periodic Acid-Schiff's Reagent
	pH 0.5	pH 2.5	pH 3.2		
scler. cut.	-	-	-	-	-
unscler. cut.	+	++	+	+	+
LPBE apolysial space	+	++	+	+	+
pLPB	+	++	+	+	+
Dcg lumen	+	+++	+	+	+
Vcg lumen	+	+++	+	+	+
per. memb.	+	++	+	-	-
Dayglo® particles*	-	-	-	-	-
food particles	+++	+++	+++	+++	+++

*; experiments using Dayglo® particles were conducted only on larvae of Aedes aegypti.

Fig. II-1. Sagittal section through head of third instar Aedes aegypti stained with alcian blue at pH 0.5. Scale bar = 200 μm.

Fig. II-2. Sagittal section through head of third instar Aedes aegypti larva stained with alcian blue at pH 3.2. Scale bar = 200 μm.

Fig. II-3. Sagittal section through head of third instar Aedes implicatus stained with alcian blue at pH 2.5. Md, mandible. Scale bar = 20 μm.

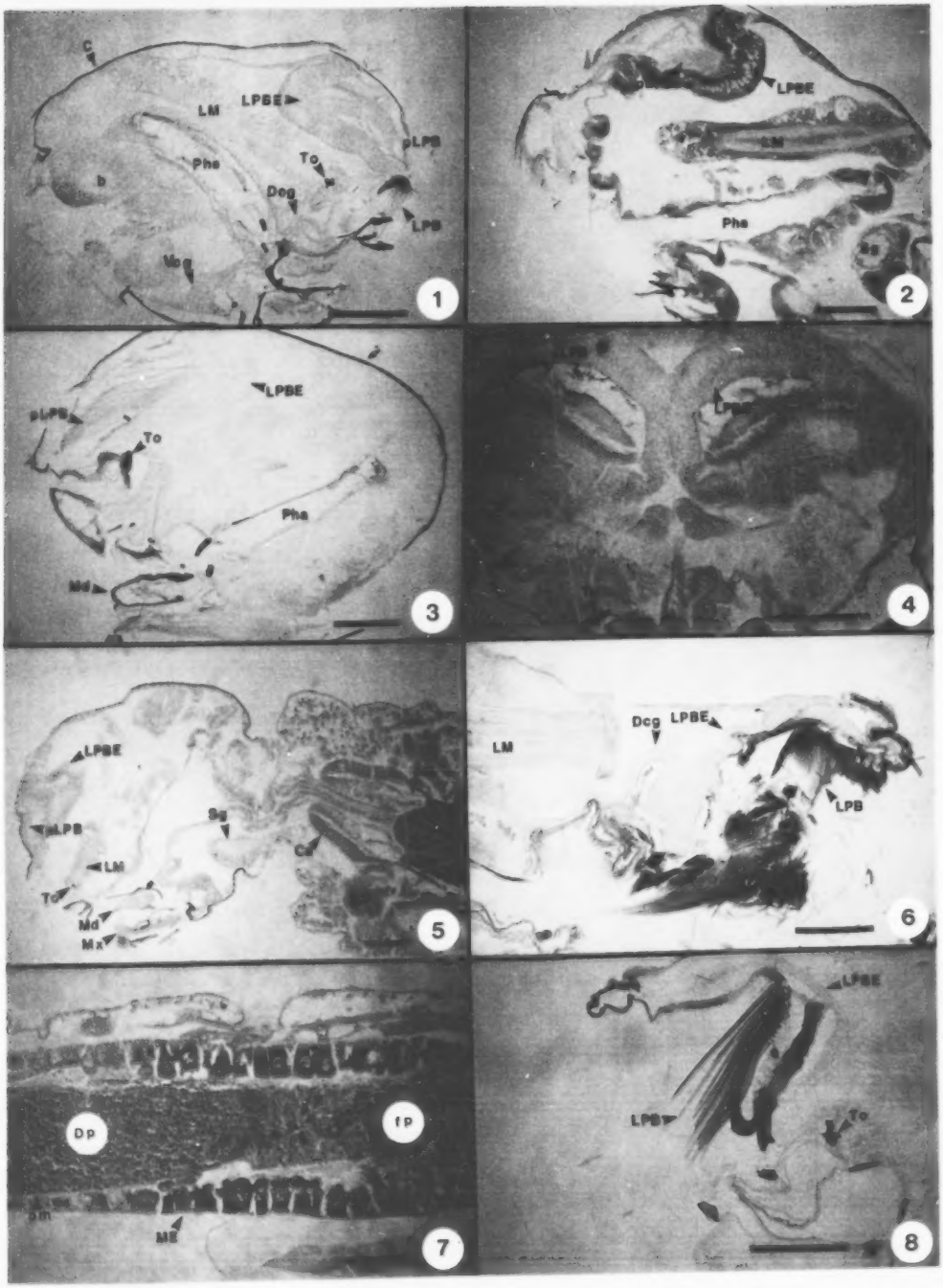
Fig. II-4. Transverse section through head of third instar Aedes aegypti stained with alcian blue and periodic acid-Schiff's reagent. Large arrowhead indicates newly-deposited cuticle. Scale bar = 200 μm.

Fig. II-5. Sagittal section through head and anterior thorax of third instar Aedes aegypti stained with alcian blue and aldehyde fuchsin. Scale bar = 200 μm.

Fig. II-6. Sagittal section through anterior head of post-teneral, third instar Anopheles earlei larva stained with alcian blue and aldehyde fuchsin. Note that the LPBE has not apolysed at this point in the larval stadium. Scale bar = 200 μm.

Fig. II-7. Sagittal section through midgut of third instar Aedes aegypti stained with alcian blue at pH 2.5. Note that the food particles (fp) and peritrophic membrane (pm) are alcianophilic and the Dayglo® particles (Dp) and midgut epidermis (ME) are not. Scale bar = 200 μm.

Fig. II-8. Sagittal section through anterior head of fourth instar of Culex territans stained with alcian blue at pH 2.5. Note the reduced cell size and lack of invagination of the lateral palatal brush epidermis (LPBE). Scale bar = 200 μm.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To
Md
Mx
5
Dp
fp
pm
ME
7
LPBE
LPB
To
8

III. GLYCOCONJUGATES OF THE CEPHALIC REGION IN LARVAE OF SIMULIUM VITTATUM ZETTERSTEDT AND GYMNOPAIS DICHOPTICOIDES WOOD (DIPTERA: SIMULIIDAE)

III.1 SYNOPSIS

Histochemical techniques were used to determine the origin and function of glycoconjugates in the cephalic region of larval black flies. Late instars of Simulium vittatum Zetterstedt and Gymnopais dichopticoides Wood were stained with alcian blue at pH 0.5, 2.5, and 3.2, aldehyde fuchsin, and Schiff's reagent to determine the chemical affinities of cephalic glycoconjugates. Additionally, larvae were fed inert Dayglo® particles and then sectioned and stained as above to determine the origin of glycoconjugates observed in the gut contents and on the labral fan rays. Examination showed that weakly-sulphated and carboxylated glycoconjugates secreted from what are redescribed here as dorsal and ventral cephalic glands and which are associated with the labral, mandibular, hypostomal, and maxillary epidermis, are not used to enhance food capture or handling. Instead, it is hypothesized that these glycoconjugates aid in formation of mouthpart structures during the pharate stages, or facilitate ecdysis. Glycoconjugates, previously observed in the gut and on the surface of the mouthparts are shown to be of exogenous origin, to be associated with particulate food, and may render food particles self-agglutinating. Agglutination is probably important to larvae in removing ultrafine particulate and dissolved organic matter from suspension and reforming it as larger faecal pellets.

III.2 INTRODUCTION

There exists a large body of work describing feeding behaviour and food-particle capture in simuliid larvae (Chance 1970; Craig 1974; Ross & Craig 1980; Chance & Craig 1986; Braimah 1987; Lacoursière 1992; Lacoursière & Craig 1993). Black flies have been described as feeding in any of three ways; collector-filterer, scraper (Cummins 1973), or predator (Currie & Craig 1987). Collector-filtering simuliid larvae anchor themselves to the substrate via a silken pad (Barr 1984) and alter their posture with respect to ambient flows (Chance & Craig 1986; Lacoursière 1992) to expose the labral fans to maximum flow velocity (Lacoursière & Craig 1993). Food-particle interception and capture is accomplished using the paired labral fans (Chance 1970) which are held to intercept either mainstream flow or vortices rising up the abdomen (Chance & Craig 1986). Captured ultrafine particulate organic matter (0.45 - 50 μm) (Lacoursière 1992) is removed from the labral fans by mandibular brushes and setae on the labrum when the fans are retracted into the cibarial cavity (Chance 1970). Wotton (1976) reported that colloids as small as 0.091μm can be ingested, but whether by drinking or normal feeding is uncertain.

Scraping or grazing by simuliid larvae has been documented as occurring in both obligate scrapers and collector-filtering larvae (Chance 1970). Fanless black flies, including members of the genera Twinnia Stone & Jamnback and Gymnopais Stone, are obligate scrapers which scrape material off the substrate using a stout dorsal labral brush and mandibular teeth. Currie and Craig (1987) and Crosskey (1990) suggest that the hypostoma is also involved in scraping material off of the substrate. The maxillae are used to clean the mouthparts in both scraping and filtering behaviours (Chance 1970).

Internally, glands in the cephalic region have been described by Becker

(1910), Strickland (1911), Puri (1925), Debot (1932), Grenier (1949), Chance (1970), and Ross & Craig (1980). Becker (1910) described dorsal and ventral glands in the cephalic region of Simulia (sic) and suggested the secretion product was the silk used to construct the pad larvae use to anchor themselves to the substrate. This secretion product was argued to be different from silk used to construct the puparium, which was said to originate from the salivary glands.

Strickland (1911), identifying the dorsal glands in larvae of Simulium hirtipes (Fries) as "pharyngeal glands", suggested that a "sticky" secretion product was deposited onto the epipharyngeal microtrichia and was involved in removing food from the labral fans. There was no mention of a ventral gland.

Puri (1925) described dorsal glands in Simulium noelleri Friederichs. Puri disagreed with Strickland about both the nature of the secretion product and the exit of the glands. However, Puri only discounted Strickland's observations and provided no explanation of his own. Once again, there was no mention of a ventral gland.

Debot (1932) described both dorsal and ventral cephalic glands in larvae of Simulium Latreille. However, Debot labeled the glands as "salivary glands", with what are today considered as salivary glands labeled as "glande séricigène". The "salivary" glands are figured as exiting into the cibarium at the epipharynx, much like Stickland's description of the gland exit. The ventral glands are figured as having no exit. Other than the paper's title and figure captions, there is no mention of structure or function of the glands, or their secretion products.

In a work on filter-feeding in Simulium by Fortner (1937), the dorsal cephalic glands are referred to as "labral glands" in her figure 3, a representation of a whole-mount of the head from the dorsal aspect, and as

"salivary glands" in her figure 4, a dorsal sagittal section of the head. No mention of form or function of the glands occurred in the text of the paper.

Grenier (1949) also described both dorsal and ventral cephalic glands in larvae of Simulium. Grenier agreed with Strickland about the function of the dorsal glands, which Grenier termed "dorsal cephalic glands". Grenier described duct-like openings for the dorsal cephalic glands, but admitted the ducts were minuscule and extremely difficult to prepare for others to identify. The ventral cephalic glands, figured as arising more ventral than those described by Debot, lacked an opening to the cibarial cavity and were presumed to secrete an elastic cuticle to aid the movement of the prementum as it slid over the hypostoma. Grenier speculated that the secretion product was similar to that produced by what he described as glandular tissue present in the anal disk and similar tissues in abdominal suckers of blephariceríd larvae (Grenier 1949). But the tissue he identified as glandular in the anal disk is an invagination of the epidermis to facilitate production of the anal hooks and is not glandular (Fry, pers. obs.).

Grenier reported that the contents of both the dorsal and ventral cephalic glands stained positively with Altmann's stain, a mixture of aniline and acid fuchsin. Although the reaction of the dorsal and ventral glands was similar, Grenier suggested that different secretion products arose from each gland: silk dorsally,.and cuticle ventrally.

Chance (1970) describes "dorsal and ventral" glands in Simulium vittatum Zetterstedt, stating that the dorsal glands emptied directly into the cibarium. No exit was observed for the ventral glands. Chance did not suggest a function for the glands, or their secretion product.

Ross and Craig (1980), using histochemical techniques, identified dorsal and ventral glands in simuliid larvae and discussed the role of mucus in food-

particle capture. They reported that a non-sulphated acid glycoconjugate was produced by the dorsal and ventral glands and suggested that it was secreted onto the labral fans to aid in particle capture. To further confuse the matter, Crosskey (1990), in reporting on the work of Ross and Craig, figured the dorsal glands and labeled them as "labral" glands (cf. Fig. 7.1(a)).

Paired dorsal and ventral glands have been described in culicid larvae (Merritt & Craig 1987). However, their reported purpose of producing a mucosubstance to aid in food-particle capture has been disputed (Dahl et al. 1988; Dahl et al. 1990; Fry & McIver 1990). Recent work has shown that newly-described dorsal and ventral cephalic glands, different from those described by Merritt and Craig, secrete a glycoconjugate which may serve a role in the moulting process (Fry in manus).

With simuliids and culicids closely related with regard to mouthpart structures and feeding system (Wood 1978), and taxonomy (Wood & Borkent 1989), it is reasonable to assume that the dorsal and ventral cephalic glands present in black flies serve a role similar to that of the glands in culicids. With the putative role and presence of mucus in particle capture so unresolved, my goal in this project was to histochemically determine the origin and function of glycoconjugates in the cephalic region of larval black flies.

The term “mucus” implies a function as a viscous material for particle capture (Jørgensen et al. 1984), or lubrication (Clamp et al. 1978; Denny & Gosline 1980). Because the function of the so called mucus in blackfly larvae is so uncertain, the chemically accurate and functionally neutral term “glycoconjugate” is used instead.

III.3 MATERIALS AND METHODS

One hundred and thirty late instars of Simulium vittatum Zetterstedt were collected from Stauffer Creek, Alberta during the summer of 1990. Thirty-one late instars of Gymnopais dichopticoides Wood were collected from a seep emanating from glacial runoff from Angel Glacier, Jasper National Park, Alberta (Canadian Parks Service Collection Permit No. 57) in July, 1990. G. dichopticoides was examined to test whether glycoconjugates function also in scraping- or grazing-type food acquisition or are restricted to the collector-filtering method of feeding. The larvae were fixed in alcoholic Bouin's for 24 hours, dehydrated in a graded ethanol series, embedded in TissuePrep® paraffin wax (melting point 56-57°C) (Fisher Scientific Co.), and sectioned at 10 µm on a Reichert-Jung rotary microtome. Serial sections were stained with alcian blue at pH 1.0, 2.5, and 3.2 (Bancroft and Stevens 1990), with alcian blue pH 2.5-aldehyde fuchsin (Spicer et al. 1962), and with alcian blue pH 2.5-periodic acid-Schiff's reagent (Mowry 1963)(Table III-1). Alcian blue electrostatically binds to polyanions bearing sulphate or carboxyl groups depending upon the ionization state of the parent sugar residues (Bancroft and Stevens 1990), and is widely accepted as an indicator of glycoconjugates (Cook 1982). Schiff's reagent, which binds to neutral glycoconjugates after periodic acid treatment, and aldehyde fuchsin, which binds to sulphated glycoconjugates, complement alcian blue staining by competing for binding sites, leaving alcian blue to stain carboxyl groups ionized at pH 2.5.

Control specimens consisted of 10 µm sections of Bouin's-fixed, paraffin embedded, human colon biopsy material and 5 µm sections of Bouin's-fixed, paraffin embedded, Trichoplusia ni (Hübner) larvae (Lepidoptera: Noctuidae). Mucus in the goblet cells of the human colon samples provided a positive control for alcian blue staining (Allen 1978). The noctuid samples, fed a known

diet (B.A. Keddie, personal communication), provided invertebrate material for comparison of gut content and peritrophic membrane staining reaction.

A test to determine the origin of glycoconjugates in larval blackfly gut contents was conducted using Dayglo® particles (Dayglo Color Corporation, Cleveland, Ohio). Dayglo® particles are chemically inert to alcian blue, Schiff's reagent and to aldehyde fuchsin (unpublished results). Fifty late instars of S. vittatum, reared in water collected at the Stauffer Creek collection site containing algae, bacteria, fine particulate and dissolved organic matter, and diatoms, were transferred to a circular 2 L tank (Craig 1977) containing distilled water and a suspension of

0.5 g of Dayglo® particles. Flow was maintained using a magnetic stir bar located in a central well of the tank resulting in a water velocity of 15-20 cm/s. To serve as a feeding stimulant (Dadd 1970; Aly 1983), 0.5 g of brewer's yeast was suspended in 500 ml distilled water, heated to 60°C, allowed to cool to room temperature, and filtered through a 0.22 µm millipore syringe filter. Twenty-five ml of filtrate were added to the Dayglo® tank. The larvae were allowed to feed on Dayglo® particles for 20 minutes and were then processed and stained as above (Table III-1). Any staining observed in the region of the gut containing Dayglo® particles would have had to originate from within the larva and not an exogenous source. Alternatively, if no staining was observed in the region of the gut containing Dayglo® particles, then staining in the region without Dayglo® particles must be due to material of exogenous origin.

Specimens examined under a Cambridge S150 scanning electron microscope were processed as above up to embedding in paraffin. Instead, larval heads were critical point dried, mounted on stubs using sticky tape, and sputter-coated with gold. Various accelerating voltages were used.

Serial sections of all material were examined using a Reichert-Jung

Polyvar widefield photomicroscope and recorded on Kodak Ektachrome T160 colour film.

Morphological terms follow those of Harbach and Knight (1980).

III.4 RESULTS

The results of staining were similar for S. vittatum and G. dichopticoides (Table III-2).

Larvae stained with alcian blue showed uniformly weak alcianophilia at pH 0.5 (Fig. III-1) and 3.2 (Fig. III-2) and strong alcianophilia at pH 2.5 (Fig. III-3) in newly-deposited, unsclerotized cuticle throughout the head capsule and mouthparts (for example pLF). Newly-forming cuticle (Fig. III-4 pLB) was periodic acid-Schiff's reagent (PAS) positive. Apodemes and tracheal cuticle exhibited weak to strong alcianophilia. Wherever muscle inserted (To), broad bands of material between the muscle and insertion point were weakly alcianophilic at pH 0.5 and 3.2, strongly alcianophilic at pH 2.5, and PAS positive (Figs. III-3, III-4). The apolysial space enclosed by the labral fan epidermis posteriorly, laterally and dorsally, the fan stem and labral fan anteriorly, and epidermis ventrally contained flocculent material exhibiting weak alcianophilia at pH 0.5 and 3.2 and strong alcianophilia at pH 2.5 (Figs. III-1–III-3). Similar material lying in the space created by apolysis in the mandibles, maxillae, labiohypopharynx, pharynx, and epipharynx were variously alcianophilic. Aldehyde fuchsin staining was evident as a dark purple to blue color wherever alcian blue pH 2.5 staining was observed in other sections. This result was expected as aldehyde fuchsin has an affinity for sulphated glycoconjugates. Unfortunately, the dark staining by aldehyde fuchsin masked any reaction of alcian blue to carboxylated glycoconjugates.

The peritrophic membrane (pm) was weakly alcianophilic at pH 0.5 and 3.2 and positively alcianophilic at pH 2.5 (Table III-2, Figs. III-1–III-3). The alcianophilia of the peritrophic membrane was uniform from point of secretion at the midgut to faecal pellet formation in the rectum. The neuropile of the brain

(b), subaesophageal ganglion (Sg), thoracic ganglia, and the ventral nerve cord were weakly alcianophilic at pH 0.5 and 3.2 and mildly alcianophilic at pH 2.5 (Figs. III-1–III-3).

Paired invaginations of epidermis lateral and posterior to the hypostoma are here redescribed as ventral cephalic glands (Figs. III-1, III-3, Vcg). The lumina of the ventral cephalic glands were continuous with apolysial space resulting from invagination or apolysis of epidermis lining the hypostoma, labiohypopharynx, mandibles and maxillae. Flocculent material in the lumina of the glands was weakly alcianophilic at pH 0.5 and 3.2 and strongly alcianophilic at pH 2.5 (Figs. III-1, III-3). There was no difference between the reaction of material in the lumina from that between the cuticle and epidermis of the mouthparts at each pH level used. When stained with PAS, areas of alcianophilia associated with the ventral cephalic glands were slightly purple, indicating the presence of neutral glycoconjugates.

Small paired invaginations of epidermis, ventro-medial to the labral fan epidermis, are here redescribed as the dorsal cephalic glands (Figs. III-1–III-4, Dcg). Flocculent material observed in the lumina of the dorsal cephalic glands was weakly alcianophilic at pH 0.5 and 3.2 and strongly alcianophilic at pH 2.5 (Figs. III-1–III-3). When stained with PAS, areas of alcianophilia associated with the dorsal cephalic glands were purple, indicating the presence of neutral glycoconjugates. However, the material in the apolysial spaces was more purple than in the lumina of the glands or newly deposited cuticle (Fig. III-5). The lumina of the dorsal cephalic glands are continuous with the apolysial space resulting from apolysis of epidermis of the labral fan.

Gut contents exhibited variable alcianophilia. Unidentifiable flocculent material (fp) exhibited variable alcianophilia (Fig. III-6). Diatom tests remained unstained. There was significant staining of gut content material by aldehyde

fuchsin. In particular, the outer coating or layer of some algae and unidentified material stained uniformly purple with aldehyde fuchsin. A varying reaction to PAS was seen in the gut contents.

Dayglo® particles (Dp) in the fore-, mid-, and hindgut did not stain with alcian blue, aldehyde fuchsin, or Schiff's reagent (Fig. III-6). There was no staining evident between the particles. Material in the gut posterior to Dayglo® particles stained variably with alcian blue, aldehyde fuchsin, or Schiff's reagent (Fig. III-6, fp), similar to the stain reaction of gut contents of non-Dayglo®-fed larvae . All other structures reacted similarly to non-Dayglo® fed larvae.

Goblet cells in the human colon specimens reacted positively to alcian blue at pH 0.5, 2.5, and 3.2. Some gut contents and the entire peritrophic membrane of T. ni reacted positively to alcian blue in a manner similar to that of the black fly species tested.

Close examination by bright-field and by Nomarski interference contrast microscopy at x1250 and by scanning electron microscopy at x25,000 of cuticular areas adjacent to the dorsal and ventral cephalic glands failed to reveal any recognizable ductule or pore system exiting into the buccal cavity.

III.5 DISCUSSION

Alcian blue at varying pH, aldehyde fuchsin, and Schiff's reagent, were used to investigate the chemical nature of glycoconjugates present in the cephalic region of blackfly larvae. Alcianophilia was observed at sites of cuticle deposition (Figs. III-1–III-5). A positive reaction to PAS in areas of cuticle deposition was indicative of neutral glycoconjugates (Fig. III-5). Based on reaction to alcian blue at varying pH levels, it appears that glycoconjugates in the apolysial spaces formed between cuticle and apolysed epidermis are weakly-sulphated, with some material being strongly-sulphated or carboxylated (Table III-2).

Material in the lumina of the dorsal and ventral cephalic glands exhibited strong alcianophilia and stained more intensely than material in the apolysial spaces. Glycoconjugates in cytoplasmic vesicles may be superconcentrated and become progressively hydrated and diffuse after discharge from the cell (Allen 1983). This might explain the difference in staining intensity between the dorsal and ventral cephalic gland lumina and the apolysial spaces.

The processes resulting in formation of the mouthparts, and especially the labral fans in simuliids and lateral palatal brushes in culicids, are extraordinary (Craig 1974; Fry & McIver 1990; see Ch. II for a more detailed explanation of moulting in culicids). Immediately following ecdysis, in all instars except the last, the epidermis underlying labral structures apolyses from the cuticle to allow for formation of the labral structures of the following instar. This is in striking contrast to the late stadium apolysis of non-elaborated cuticle such as that of the head capsule and thoracic and abdominal sclerites. In simuliid larvae, a large apolysial space develops between the labral cuticle and the apex of the epidermal cells. This space, filled with material of unknown origin and function, is continuous with that between the head capsule and epidermis

throughout the head. Similarly, apolysial spaces develop in the mandibles and maxillae.

Material found in the apolysial spaces may arise from the dorsal and ventral cephalic glands. The lumina of the cephalic glands are continuous with these spaces. Further, the absence of a pore or ductule system for glycoconjugates to exit to the cibarial cavity or pharynx, and the lack of staining in the gut region containing Dayglo® particles indicates that material arising from the dorsal and ventral glands is not used to aid particle capture or handling.

Exopolymers associated with microorganisms (Decho & Moriarty 1990) or flocculent dissolved organic matter may be sources of glycoconjugates responsible for agglutination or aggregation of both coarse and fine particulate organic matter (CPOM and FPOM, respectively) (Merritt et al. 1992b; Ward et al. 1990). A similar phenomenon appears to occur with ultrafine particulate organic matter (UPOM) (0.45 - 50 μm), the particle size range collected by black flies (Lacoursière & Craig 1993). Some CPOM and FPOM, uncolonized by microorganisms, may have labile sugars coating particle surfaces (Wotton 1990a), which render them sticky. This apparent self-agglutinating character of ingested food may account for food compaction in the pharynx and foregut of simuliids. It follows that the alcianophilia of glycoconjugates observed in the gut is now highly likely due to glycoconjugates associated with food items (Fig. III-6). The lack of alcianophilia in the region of gut containing Dayglo® particles supports the hypothesis of no endogenous glycoconjugate production for use in food capture or handling. Faecal pellets may remain intact because of undigested glycoconjugates originating from food material, in addition to the enveloping, sleeve-like peritrophic membrane. Wotton (1978) reported that absorption efficiencies in blackfly larvae were as low as 1.75%. Incomplete

absorption of nutrients, and rapid transport of gut contents may also contribute to preserving the integrity of faecal pellets (Clements 1992; Wotton 1988).

Food-particle size is altered during feeding by blackfly larvae. UPOM and FPOM is incorporated into larger faecal pellets which may be more suitable for grazers or other collector-filterers (Ward et al. 1990). Food size alteration is an example of one mechanism of 'nutrient spiraling' (Webster & Patten 1979) that may involve simuliid larvae reintroducing food to the ecosystem in a form more attractive or available to other organisms.

Collector-filterers, such as simuliids, may be instrumental in retaining seston which sediments out very slowly (McCullough et al. 1979). Morin et al. (1988) reported an 8.7% drop of seston downstream of black flies at a lake outlet. Merritt et al. (1992a) reported that Anopheles quadrimaculatus removes particulate food from the surface layers and incorporates it into a downward-directed lamellate plume of water. Alternatively, bottom-feeding aedine larvae may dislodge biofilm or exopolymeric material and resuspend it in the water column for collector-filterers (Wotton 1990b).

In confined or restricted habitats, repositioning food resources may have a significant impact on intraspecific competition (Aly 1988). Coprophagy, either directly by grazing or indirectly by intercepting resuspended faecal pellets may modify larval distribution in the microhabitat. However, Ciborowski and Craig (1989) found larval spacing to be unaffected by food availability except at low food concentrations when lateral alignment of the larvae was observed.

There is no apparent role for endogenously produced glycoconjugates in food capture or handling in collector-filterer simuliid larvae. Additionally, glycoconjugates arising from dorsal and ventral cephalic glands are present in G. dichopticoides, an obligate scraper. There should be a function common to both collector-filterers and obligate scrapers for the secretion product of the

cephalic glands. Therefore, an alternative hypothesis is required to explain the function of glycoconjugates produced by the dorsal and ventral cephalic glands in simuliids.

The glycoconjugates observed may function in the moulting process. Glycoconjugates may act to support the anteromedian palatal brush in Gymnopais, labral fan in Simulium, and the various brushes and setae of the mandibles and maxillae in both as these structures develop in the large apolysial space. In particular, the labral fan is a relatively long structure that develops inside the head capsule but outside the epidermis and requires support during each larval stadium. For such a large structure to be supported and possibly immobilized during its development, the fluid filling the apolysial space, comprised in part of glycoconjugates, would have to exhibit qualities similar to that of an extracellular matrix. The glycoconjugates observed may be a form of extracellular matrix where the local environment surrounding developing labral fan rays could be of a highly ordered state to facilitate growth of the fan ray and deposition of cuticle. The disturbance or possible contact between rays resulting from normal feeding activity where hydrostatic pressure is exploited to adduct the labral fans could be ameliorated by such a viscous medium characteristic of glycoconjugate-dominated fluids.

The apolysial space is filled and it seems unlikely to be an external fluid or haemolymph. To have the apolysial space filled from the external environment would risk infection by bacteria, viruses or parasites. Additionally, no pore or duct system was observed to allow passage of fluid from the external environment into the apolysial space. The dorsal cephalic glands may be the source of glycoconjugates which provide the support. Similarly, the ventral cephalic glands may supply the material filling apolysial spaces associated with the ventral mouthparts. The reduced size of these glands, compared to the

dorsal cephalic glands, may be indicative of the smaller volume of apolysial spaces requiring material ventrally.

Results from identical tests on several species of mosquitoes were similar to those found for S. vittatum and G. dichopticoides (see Chapter II). Although mosquitoes inhabit still waters, many of the same challenges are faced in food acquisition. That members of two different families exhibit similar solutions to a common problem, that of accomodating development of elaborate cephalic structures, lends support to the hypothesis presented to explain the presence of glycoconjugates in both families.

Although the use of glycoconjugates in food-particle capture during filter feeding by larval black flies has now been refuted, their role in larval development requires further study. The precise composition of glycoconjugates secreted by the dorsal and ventral cephalic glands remains unknown. The use of sensitive and precise cytochemical techniques to determine the composition of the glycoconjugates will facilitate understanding of their role in larval blackfly development.

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TABLE II-1. APPORTIONMENT OF BLACK FLY LARVAE STAINED WITH ALCIAN BLUE, ALDEHYDE FUCHSIN, AND PERIODIC ACID-SCHIFF'S REAGENT

Species	Stain				
	Alcian Blue			Aldehyde	Periodic
	pH 1.0	pH 2.5	pH 3.2	Fuchsin	Acid-Schiff's Reagent
Simulium vittatum	20	19	10	19	12
Gymnopais dichopticoides	6	7	6	6	6

TABLE III-2. GLYCOCONJUGATE STAINING IN LARVAE OF SIMULIUM VITTATUM AND GYMNOPAIS DICHOPTICOIDES

Structure	Alcian blue			Aldehyde Fuchsin	Periodic Acid-Schiff's Reagent
	pH 0.5	pH 2.5	pH 3.2		
scler. cut.	-	-	-	-	-
unscler. cut.	+	++	+	+	+
LF apolysial space	+	++	+	+	+
pLF	+	++	+	+	+
Dcg lumen	+	+++	+	+	+
Vcg lumen	+	+++	+	+	+
per. memb.	+	++	+	-	-
Dayglo® particles*	-	-	-	-	-
food particles	+++	+++	+++	+++	+++

*; experiments using Dayglo® particles were conducted only on larvae of S. vittatum.

Fig. III-1. Longitudinal section through the head and anterior segment of thorax of a third instar Gymnopais dichopticoides stained with alcian blue at pH 0.5. Scale bar = 100 μm.

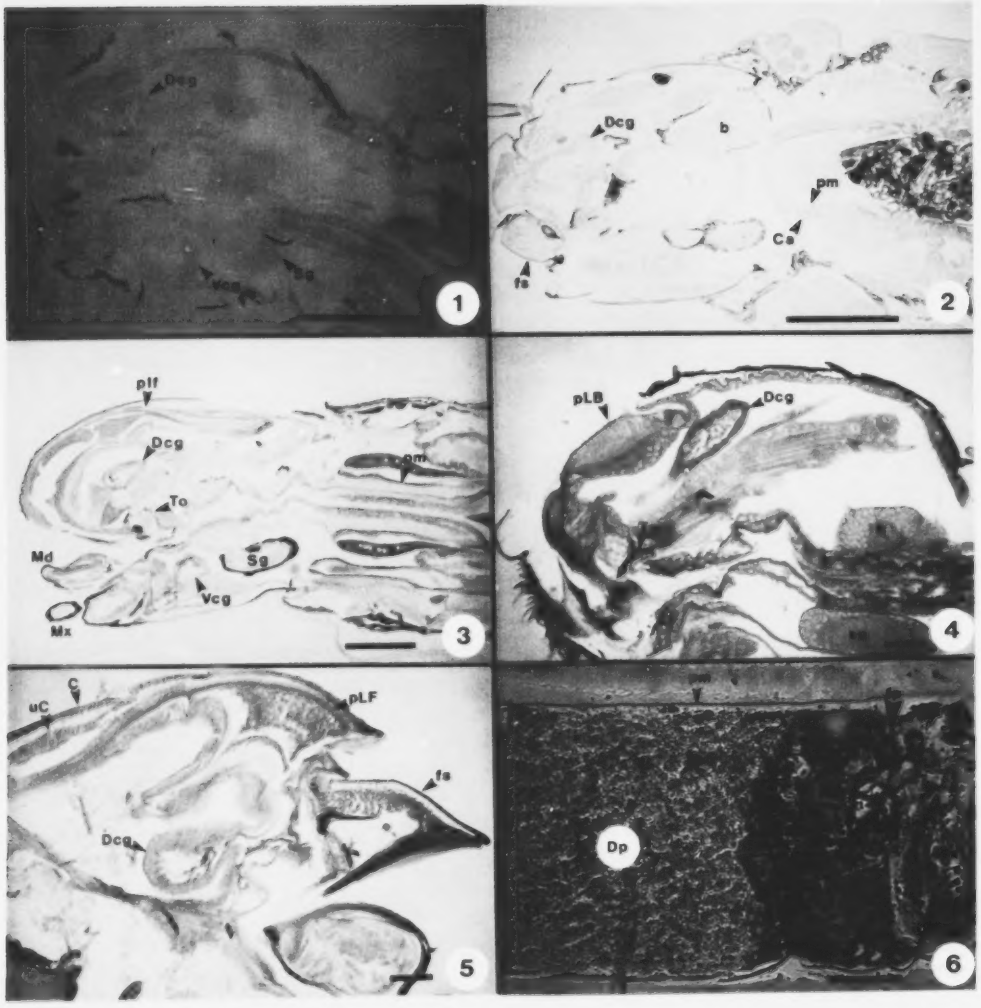
Fig. III-2. Horizontal section through the head and anterior two segments of the thorax of a third instar Simulium vittatum stained with alcian blue at pH 3.2. Scale bar = 100 μm.

Fig. III-3. Longitudinal section through the head and anterior two segments of thorax of a third instar Simulium vittatum stained with alcian blue at pH 2.5. Scale bar = 50 μm.

Fig. III-4. Longitudinal section through the head of a third instar Gymnopais dichopticoides stained with alcian blue at pH 2.5 and periodic acid-Schiff's reagent. Scale bar = 10 μm.

Fig. III-5. Longitudinal section through the head of a third instar Simulium vittatum stained with alcian blue at pH 2.5 and periodic acid-Schiff's reagent. Scale bar = 10 μm.

Fig. III-6. Longitudinal section through the midgut of a third instar Simulium vittatum stained with alcian blue at pH 2.5. Note that the food particles (fp) and peritrophic membrane (pm) are alcianophilic and the Dayglo® particles (Dp) and midgut epidermis (ME) are not. Scale bar = 10 μm.


Dcg
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Dp
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IV A PRELIMINARY STUDY OF THE CEPHALIC REGION OF AEDES AEGYPTI (L.) LARVAE (DIPTERA: CULICIDAE) USING LECTINS

IV.1 SYNOPSIS

Lectins derived from Triticum vulgaris or wheat germ agglutinin (WGA) specific for N-acetyl-β-D-glucosamine, Bandeiraea simplicifolia (BS I)specific for α-D-Galactose, Sophora japonica (SJA) specific for N-acetyl-β-D-galactose, Pisum sativum (PSA) specific for α-D-mannose, and Ulex europaeus (UEA I) specific for α-L-fucose were used to determine the composition of the secretion product arising from the dorsal and ventral cephalic glands in larvae of Aedes aegypti (L.). With the exception of PSA, none of the lectins bound to the secretion product or glands. Both globular and elongate cells in the ventral cephalic gland bound PSA, indicating the presence of α-mannose. Agglutinin from Bandeiraea simplicifolia bound to the lateral palatal pennicular area, the dorsal surface of the hypostome, the bases of large brush-like structures on the anteromedial palatum and maxillae. This reaction may indicate the presence of resilin. Agglutinin from Triticum vulgaris bound to unsclerotized cuticle throughout the head.

The composition of the secretion product of the dorsal and ventral cephalic glands remains undetermined. In depth analysis using lectins with substrate specificities different than the ones used in this study, coupled with digestion techniques, may yield greater resolution of the secretion product.

IV.2 INTRODUCTION

Lectins have been used to detect glycoconjugates in several different tissues in many taxa (Goldstein & Hayes 1978; Sharon & Lis 1989). A lectin's affinity for highly specific binding to a substrate potentially affords extreme accuracy in determining carbohydrate content in tissue. It was with this property in mind that the problem of the composition of secretion products of dorsal and ventral cephalic glands in culicid larvae was investigated using selected lectins with different substrate specificities.

In an investigation of feeding systems in larval culicids and simuliids (Fry, in manus), the dorsal and ventral cephalic glands secretion product, and associated material in mouthpart apolysial spaces, reacted positively to carbohydrate staining techniques. The secretion product was hypothesized to serve a function in moulting instead of use in food-particle capture or manipulation as suggested by Merritt and Craig (1987). To acquire greater insight into the role of the gland material a more detailed description of the composition of the secretion product is required.

The purpose of this study was to determine, in a preliminary manner and using lectins of different specificities, the carbohydrate content of secretion products of the dorsal and ventral cephalic glands and of material found in mouthpart apolysial spaces.

IV.3 MATERIALS AND METHODS

Third instar Aedes aegypti (L.) were obtained from a laboratory colony maintained in the Department of Entomology, University of Alberta. Larvae were fixed in aqueous Bouin's for 24 hours, dehydrated in a graded ethanol series, embedded in TissuePrep® paraffin wax (melting point 56-57°C) (Fisher Scientific Co.), and sectioned at 7 μm on a Reichert-Jung rotary microtome. Aqueous Bouin's was used as the fixative to preserve continuity in specimen reactivity between specimens analyzed as described in chapters II and III and to strike a compromise between preserving substrate reactivity and preservation of strucutre.

Larval material was stained with one of 5 lectin solutions (Table IV-1):

1. Agglutinin derived from Triticum vulgaris, also known as wheat germ agglutinin (WGA), conjugated to fluorescein isothiocyanate (green at 525nm) (FITC) with specificity for N-acetyl-β-D-glucosamine (β-GlcNAc) (Allen et al. 1973). β-GlcNAc is the principle component of chitin (Gillott 1991).

2. Agglutinin derived from Bandeiraea simplicifolia (BS I) conjugated to tetramethylrhodamine isothiocyanate (red at 710nm) (TRITC) with specificity for α-D-Galactose (α-Gal) and N-acetyl-α-D-galactosamine (α-GalNAc) (Hayes & Goldstein 1974). α-GalNAc is one anomeric form of N-acetyl-D-galactosamine.

3. Agglutinin derived from Sophora japonica (SJA-FITC) with specificity for N-acetyl-β-D-galactose (β-GalNAc) (Wu et al. 1981). β-GalNAc is a common constituent of vertebrate mucus (Clamp et al. 1978)

4. Agglutinin derived from Pisum sativum (PSA-TRITC) with specificity for α-D-mannose (α-Man) (Van Wauwe et al. 1975). α-Man has been associated with mucus (Spicer & Schulte 1992).

5. Agglutinin derived from Ulex europaeus (UEA I-FITC) with specificity

for α-L-fucose (α-Fuc) (Matsumoto & Osawa 1969). α-Fuc has been associated with mucus (Spicer & Schulte 1992).

All lectins were prepared in tris-HCl buffer (pH 7.2) containing sodium chloride, magnesium chloride, calcium chloride, and manganese chloride to enhance binding of lectin to the substrate (Mauchamp & Schrével 1977), and Triton X-100 detergent to reduce non-specific hydrophobic binding. Lectins were used at a final concentration of 10 µg/ml.

Biopsy samples of human colon, prepared as above, were used for controls (Clamp et al. 1981). Additionally, lectins were incubated in 10,000 µg/ml of the appropriate substrate for 1 hr prior to staining insect material as a test of substrate specificity (Table IV-1). Substrates used were: N-acetyl-D-glucosamine for WGA, D(+)-galactose for BS I, N-acetyl-D-galactosamine for SJA, D(+)-mannose for PSA, and α-L(-)-fucose for UEA I. All lectins and substrates were obtained from Sigma® Chemical Co., St. Louis.

Sections were viewed on a Polyvar fluorescence photomicroscope and recorded on Kodak Ektachrome 400HC color slide film. Larval structure nomenclature follows that of Harbach and Knight (1980).

IV.4 RESULTS

Results for A. aegypti are summarized in Table IV-2.

With WGA, positive staining of newly deposited unsclerotized cuticle of the head capsule (C), pharynx (Pha), epipharynx (ep), lateral palatal pennicular area (a reticulated area of cuticle dorsal to the lateral palatal brush), anteromedian palatal brush, and ventral mouthparts was observed (Fig. IV-1). Tracheoles and the torma also reacted positively to WGA. However, the torma (the apodeme upon which the labral adductor muscles insert), tracheoles, pharate epipharyngeal, and pharate pharyngeal cuticle remained positive after staining with substrate-incubated WGA (Fig. IV-2). Additionally, the lateral palatal pennicular area and anteromedian palatal brush stained only weakly with substrate-incubated WGA. Pharate head capsule cuticle reacted weakly to substrate-incubated WGA.

BS I stained positively the base of the epipharynx, the fold of cuticle between the mandibles and maxillae, tracheoles, and the dorso-proximal surface of the hypostome (H) (Fig. IV-3). The lateral palatal pennicular area (LPPA), torma, pharynx, maxillary brush base (MxBr), and apex of the lateral palatal brush filaments (LPB) also reacted to BS I (Fig. IV-4). Only the pharynx reacted to substrate-incubated BS I (Fig. IV-5, arrowhead).

There was no apparent staining of any structures by SJA (Fig. IV-6). Substrate-incubated SJA bound to the pharate head caspsule (C) and pharyngeal cuticle and cuticle at the base of the developing lateral palatal brush (pLPB) (Fig. IV-7).

PSA reacted positively to large round cells located basally, and elongate apically-narrowed cells located apically within the ventral cephalic gland tissue (Vcg), (Fig. IV-8). No other structures stained positively with PSA. A significant

reduction in staining of the cells in the ventral cephalic gland area was seen in substrate-incubated PSA sections (Fig. IV-9).

With UEA I, the only structure to show a positive result was the torma (To) and this reaction was negligible to weakly positive in intensity (Fig. IV-10). As with SJA, pharate head capsule (C) and pharyngeal cuticle, and cuticle ventral to the LPB reacted positively to substrate-incubated UEA I (Fig. IV-11).

The salivary duct contents (Fig. IV-1, sd) were autofluorescent in all treatments and controls.

IV.5 DISCUSSION

Specific lectins were used to investigate the chemical nature of material secreted from dorsal and ventral cephalic glands in larval mosquitoes. The lectins used have different specificities including those for sugar moieties common to cuticle, N-acetyl-glucosamine, and mucus, N-acetyl-galactosamine. With the exception of PSA, none of the lectins bound to material associated with the glands (Table IV-2), therefore it may be assumed that the secretion product of the dorsal and ventral cephalic glands is not composed of β-GlcNAc, α-GalNAc, β-GalNAc, α-Gal, or α-Fuc.

WGA bound more readily to newly deposited unsclerotized cuticle (Fig. IV-1) than did the substrate-incubated lectin. Dörner & Peters (1988) reported on the binding of WGA to chitin in the peritrophic membrane of mosquito and black fly larvae . The binding by WGA was expected since chitin is a polymer comprised of N-acetyl-glucosamine. Binding by the substrate-incubated lectin was also expected as chitin is highly reactive to WGA (Mauchamp & Schrével 1977). No binding of tanned cuticle was observed so sclerotization must render β-GlcNAc unreactive to WGA.

Several structures were positive for galactose as indicated by BS I binding (Fig. IV-3, 4). It appears there is a peculiar specificity for sites where repeated bending, folding, or tension occurs. Perhaps these sites consist, in part, of resilin. Resilin is an elastic material consisting of amino acids, chitin, and possibly other glycoconjugates. Although resilin and membranous cuticle are not sclerotized, sclerotized cuticle is not elastic (Chapman 1982). Whitten (1972) suggested that resilin may be present in tracheoles. The basal area of large cuticular structures, such as the maxillary brush, epipharynx, pharyngeal fringe, and LPB, may be subject to stress forces requiring the increased flexibility of resilin.

Both SJA and UEA I failed to bind to any structures or material in the larval head (Figs. IV-6, 10). N-acetyl-galactose and fucose are apparently not present or were unreactive under the conditions of staining. The lack of β-GalNAc, a common constituent of human mucus (Clamp et al. 1978) supports the hypothesis that the secretion product of the dorsal and ventral glands is not involved in particle capture or handling (Fry, in manus).

The reaction of cells in the ventral gland to PSA (Fig. IV-8) indicates the presence of α-Man. No reaction to PSA was observed in the dorsal cephalic gland. This difference between the dorsal and ventral cephalic glands needs to be investigated further. Clamp et al. (1981) claim that low-molecular weight compounds such as mannose may form cross-links with other glycoconjugates thereby conferring high viscosity on the solution. Perhaps factors such as the volume of material required or timing of secretion from the ventral cephalic gland may determine composition. Alternatively, the α-Man may be altered prior to secretion so as to be unreactive to PSA outside of the cytoplasm.

Material in the dorsal and ventral cephalic gland lumina and apolysial spaces reacted positively to alcian blue (Fry, in manus). Why material that is detectable by a well-established stain for carbohydrates fails to be detectable by the lectins used in this study may be due to several reasons. The material may be composed of anomers of the sugars tested for, for example β-Man or α-GlcNAc. The secretion product may have been rendered unreactive to the lectins by the fixative used. Alternatively, the material may be bound to a protein that masks the binding sites of the carbohydrate moiety.

A more thorough analysis, using several more lectins with various specificities, and digestion techniques to free up potentially bound reactive sites, may allow for determination of the glands secretion product. Until the

chemical nature of the secretion product is better known, understanding of its role in moulting will be limited.

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TABLE IV-1. APPORTIONMENT OF LARVAE OF *AEDES AEGYPTI* STAINED WITH LECTINS

Staining Solution	Number of *Aedes aegypti*
WGA	4
WGA + N-acetyl-D-Glucosamine	2
BS I	3
BS I + D(+)-Galactose	2
SJA	2
SJA + N-acetyl-D-Galactosamine	2
PSA	2
PSA + D(+)-Mannose	2
UEA I	2
UEA I + α-L(-)-Fucose	2
Tris buffer	3

TABLE IV-2. LECTIN-BINDING IN THE CEPHALIC REGION OF LARVAE OF AEDES AEGYPTI (L.)*

Structure	Lectin (sugar specificity)				
	WGA (β-GlcNAc)	BS I (α-Gal) (α-GalNAc)	SJA (β-GalNAc)	PSA (α-Man)	UEA I (α-Fuc)
unscler. cut.	++	-	-	-	-
LPPA	+	++	-	-	-
LPB	-	+	-	-	-
APBr	+	+	-	-	-
epipharynx	-	++	-	-	-
Pha. fringe	-	++	-	-	-
Mx. brush	-	++	-	-	-
hypostome	-	++	-	-	-
torma	-	++	-	-	-
tracheoles	-	++	-	-	-
Vcg	-	-	-	++	-
Dcg	-	-	-	-	-

*: positive reactions recorded are those after subtraction of control staining in substrate-incubated lectin

Fig. IV-1. Sagittal section through head of third instar Aedes aegypti stained with WGA-FITC. Scale bar = 20 μm.

Fig. IV-2. Sagittal section through head of third instar Aedes aegypti stained with N-acetyl-D-glucosamine-incubated WGA-FITC. Scale bar = 50 μm.

Fig. IV-3. Sagittal section through head of third instar Aedes aegypti stained with BS I-TRITC. Scale bar = 50 μm.

Fig. IV-4. Sagittal section through head of third instar Aedes aegypti stained with BS I-TRITC. Scale bar = 20 μm.

Fig IV-5. Sagittal section through head of third instar Aedes aegypti stained with D(+)-galactose-incubated BS I-TRITC. Note that only the pharyngeal fringe (arrowhead) shows a weak positive reaction. Scale bar = 20 μm.

Fig IV-6. Sagittal section through head of third instar Aedes aegypti stained with SJA-FITC. Note that only the pharynx (Pha) is positive. Scale bar = 50 μm.

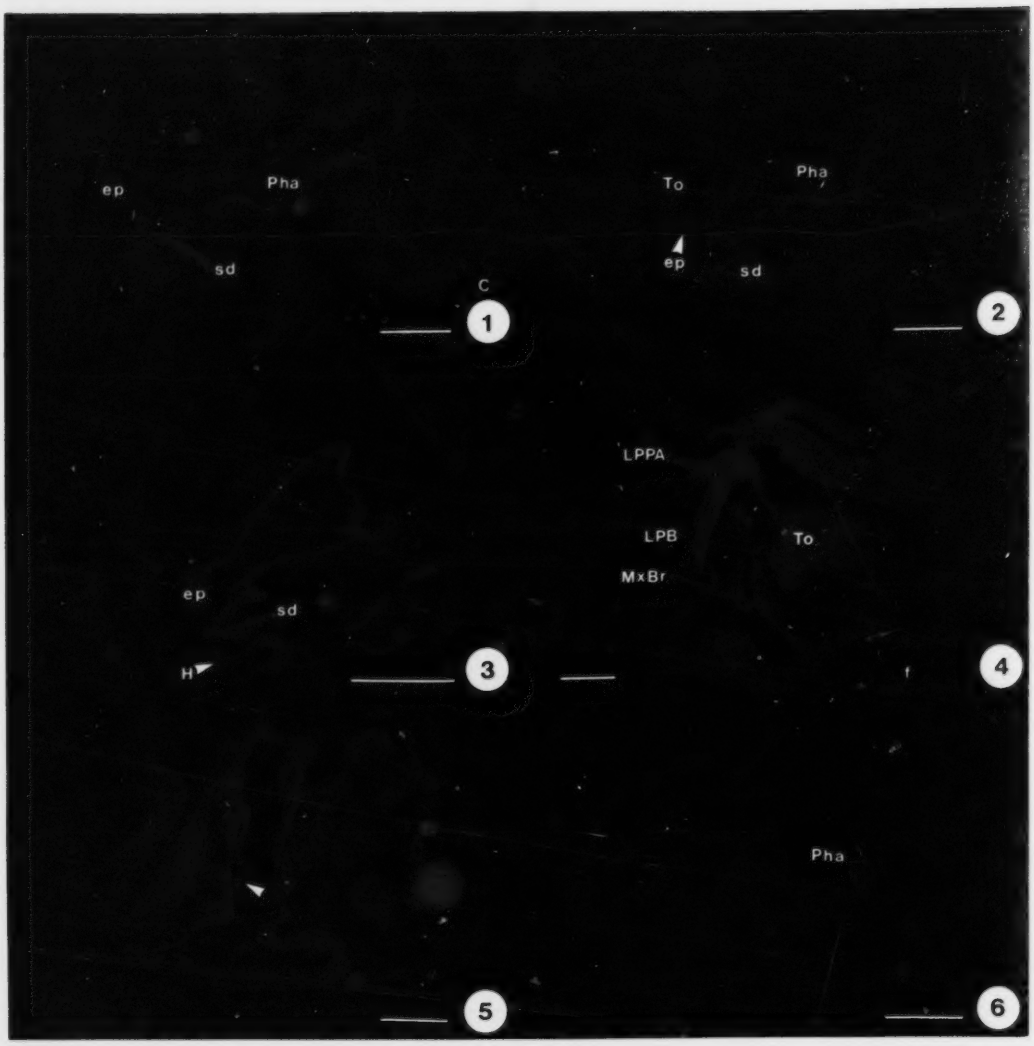

ep
Pha
To
Pha
ep
sd
sd
C
1
2
LPPA
LPB
To
MxBr
ep
sd
H
3
4
Pha
5
6

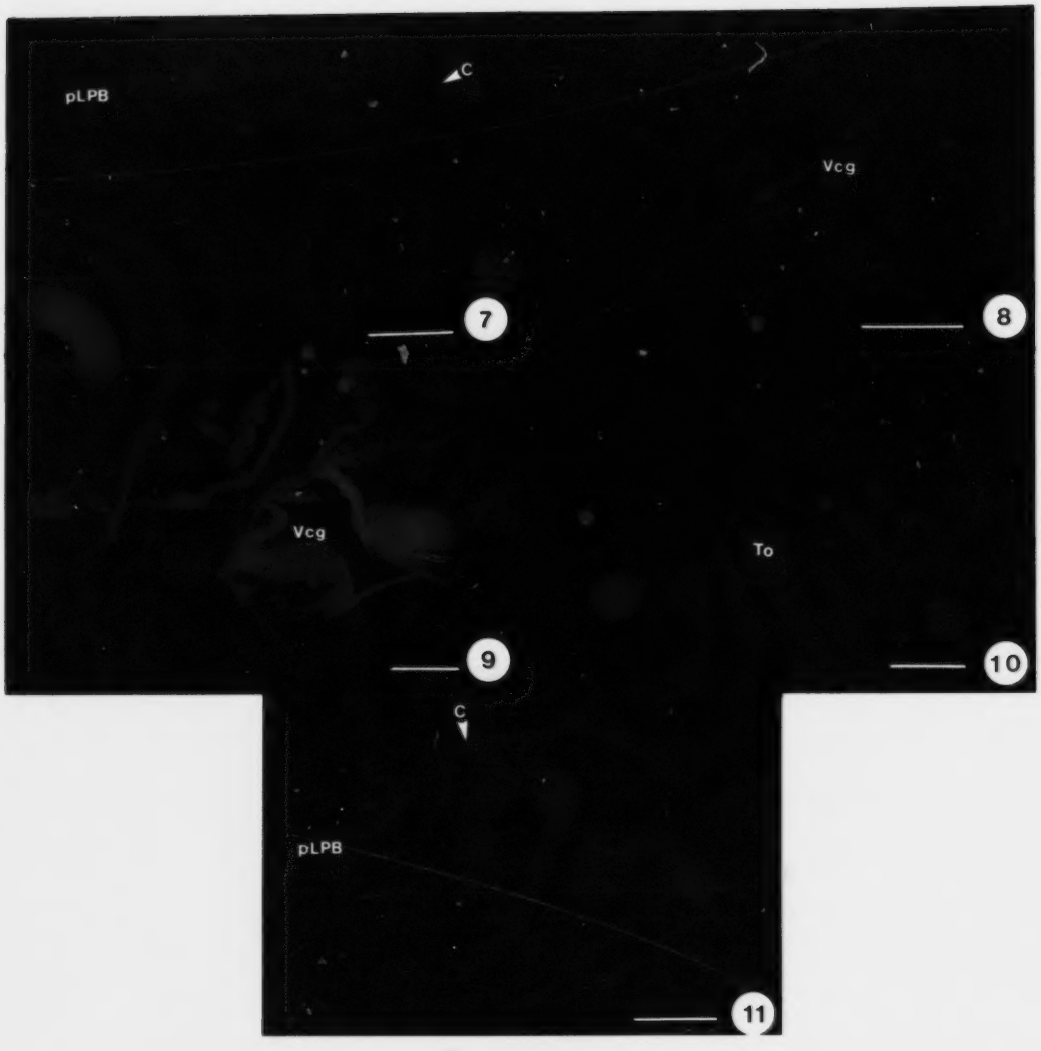
Fig. IV-7. Sagittal section through head of third instar Aedes aegypti stained with N-acetyl-D-galactosamine-incubated SJA-FITC. Scale bar = 50 μm.

Fig. IV-8. Sagittal section through head of third instar Aedes aegypti stained with PSA-TRITC. Note the cells in the ventral cephalic gland (Vcg). Scale bar = 50 μm.

Fig. IV-9. Sagittal section through head of third instar Aedes aegypti stained with D(+)-mannose-incubated PSA-TRITC. Note the lack of staining of cells in the ventral cephalic gland (Vcg). Scale bar = 50 μm.

Fig. IV-10. Sagittal section through head of third instar Aedes aegypti stained with UEA I-FITC. Note the positive reaction of the torma (To). Scale bar = 50 μm.

FIG. IV-11. Sagittal section through head of third instar Aedes aegypti stained with L(-)-fucose-incubated UEA I-FITC. Scale bar = 50 μm.


pLPB
C
Vcg
7
8
Vcg
To
9
10
C
pLPB
11

V GENERAL CONCLUSIONS

Efforts to understand the precise mechanism of feeding in black fly and mosquito larvae are beginning to branch out from classical analysis of structure to investigating physical and behavioural systems. The thrust of this project was to focus on physico-chemical influences in food-particle capture and handling. It has been found that endogenously produced glycoconjugates are not used to aid in food-particle capture or handling. Instead, the role of dissolved organic matter, glycoconjugates of planktonic origin, and the potentially self-agglutinating properties of fine and coarse particulate organic matter must be considered when modeling food-particle capture in filter-feeding organisms. Specifically, dissolved organic matter may be significantly more labile than originally believed. This fact has serious implications in evaluating feeding efficacy and in determining available nutrient resources in aquatic habitats.

With the original hypothesis regarding the role of secretory products from the dorsal and ventral glands in larval culicids and simuliids falsified, an alternative hypothesis was required to explain the presence of well-developed glandular tissue in the cephalic region and fluid in large apolysial spaces associated with developing mouthparts. It was hypothesized that, instead of secretion externally, the material originating in the dorsal and ventral cephalic glands is produced for use internally.

The development of larval culicid and simuliid mouthparts, the elaborate labrum in particular, is unlike that of head capsule cuticle or thoracic and abdominal cuticle. The large structures associated with the mouthparts require very early apolysis of the epidermis to facilitate development during the larval stadium. These structures are surrounded by material that resembles extracellular matrix material. The material in the apolysial space is

hypothesized to arise from the dorsal and ventral cephalic glands. It may serve, in the case of the labrum, as an effector for the haemocoelic hydrostatic skeleton to achieve adduction of the labral fans or brushes. There are no direct adductor muscles associated with the labral structures and it is believed that action of the haemocoel is the cause of adduction (Crosskey 1990). It is commonplace to cause the adduction of both culicid and simuliid labral structures by applying pressure to the abdomen or especially the thorax (K. Fry and others, unpublished results). The fluid may be viscous enough to provide a stable microenvironment around developing mouthparts as well as to protect the mouthparts from displacement or mechanical damage during development. Therefore, a developmental role for the secretion product of the cephalic glands may be related to the moulting process. The cephalic gland secretion product is probably an integral component of the moulting process of the relatively large mouthparts. In particular, the complex development of lateral palatal brush filaments in Aedes aegypti larvae proceeds by apical extension of the lateral palatal brush epidermal cells to form the pharate lateral palatal brush (Fry & McIver 1990). Such delicate structures are in contact with the secretion product and may derive more than physical support from the apolysial space fluid.

Efforts to determine the precise chemical composition beyond classical histochemical methods yielded more questions than answers. Apparently, the material is not comprised of sugars common to cuticle or vertebrate mucus as might be expected. Significantly more work is required to adequately determine the composition of the dorsal and ventral cephalic gland secretion products. Perhaps with an idea as to its make-up, a better understanding of its role in the moult cycle will be discovered, whether strictly as a support fluid, a storehouse of precursors, or both.

As mentioned in the introduction, diverse avenues of investigation should

be followed to finally elucidate the pertinent features of filter-feeding in aquatic larvae. With a greater understanding of an organism's habits, it should be possible to tailor control measures to impact only the target, with no effect on non-target organisms. To achieve this environmentally responsible goal, research into all areas of the biology of economically important and benign species should be supported.

V.1 LITERATURE CITED

Crosskey, R. W. 1990. The Natural History of Blackflies. John Wiley & Sons, New York.

Fry, K. M. and McIver, S. B. 1990. Development of the lateral palatal brush in larvae of Aedes aegypti (L.)(Diptera: Culicidae). Can. J. Zool. **68**: 1454-1467.

APPENDIX I FORMULAE FOR CHEMICAL SOLUTIONS

1. 0.2% Sodium Acetate

sodium acetate	2.72 g
distilled water	to 100 ml

2. 0.5% Acetic Acid

glacial acetic acid	0.5 ml
distilled water	to 100 ml

3. 0.5% Sodium Bisulphite

sodium bisulphite	0.5 g
distilled water	to 100 ml

4. 1% Periodic Acid

periodic acid	0.4 g
98% ethanol	35 ml
0.2 M sodium acetate	5 ml
distilled water	10 ml

5. 3% Acetic Acid

glacial acetic acid	3 ml
distilled water	to 100 ml

6. Alcian Blue pH 0.5

alcian blue 8GX C.I. 74240	1 g
0.2 M hydrochloric acid	to 100 ml

conc. hydrochloric acid.....≈ 1 ml

• add conc. hydrochloric acid to pH 0.5

7. Alcian Blue pH 2.5

alcian blue 8GX C.I. 74240.....1 g

glacial acetic acid.....≈ 3 ml

distilled water.....97 ml

• add glacial acetic acid to pH 2.5

8. Alcian Blue pH 3.2

alcian blue 8GX C.I. 74240.....1 g

0.5% acetic acid.....to 100 ml

glacial acetic acid.....≈ 1 ml

• add glacial acetic acid to pH 3.2

9. Alcoholic Bouin Fixative (Humason 1972)

80% ethanol.....150 ml

formalin.....60 ml

glacial acetic acid.....15 ml

picric acid crystals.....1 g

10. Aldehyde Fuchsin (Spicer et al. 1962)

pararosaniline C.I. 42500.....1 g

60% ethanol.....97 ml

conc. hydrochloric acid.....1 ml

paraformaldehyde.....2 ml

chloroform 50 ml

distilled water.....200 ml

70% ethanol.....to 100 ml

conc. hydrochloric acid.....1 ml

- dissolve pararosaniline in 60% ethanol
- add conc. hydrochloric acid and paraformaldehyde
- let solution sit for 3-4 days
- add solution to 50 ml chloroform and 200 ml distilled water in a separating funnel, agitate, and allow precipitate to settle
- drain off contents containing precipitate and filter without suction
- dry at 50°C and store in a stoppered bottle
- for use, add 0.5 g of dried material to 99 ml 70% ethanol and 1 ml conc. hydrochloric acid

11. Bouin Fixative (Humason 1972)

picric acid, saturated aqueous.....75 ml

formalin.....25 ml

glacial acetic acid.....5 ml

12. Lectin Control Solution

sugar base.....1 g

Tris working buffer.....to 50 ml

- yields 20 mg/ml of sugar base
- dilute 1:1 with lectin solution to yield 10 mg/ml sugar base

13. Neutral Red

neutral red C.I. 50040.....0.5 g

distilled water.....to 100 ml

14. Schiff's Reagent (Mowry 1963)

pararosaniline C.I. 42500.....1 g

potassium metabisulphite .....2 g

conc. hydrochloric acid.....2 ml

activated charcoal.....2 g

distilled water.....200 ml

- dissolve pararosaniline in distilled water that has been brought to a boil (remove from heat before adding chemical)
- allow to cool to 50°C
- add potassium metabisulphite
- add hydrochloric acid
- add activated charcoal, gently agitate and then leave solution overnight at room temperature in the dark
- agitate solution and then filter through Whatman® #1 filter and store in the dark at 4°C

15. Tris Salt Buffer Stock Solution

tris(hydroxymethyl)aminomethane hydrochloride.....6.057 g

sodium chloride.....8.7 g

magnesium chloride.....0.203 g

manganese chloride .....0.198 g

calcium chloride.....0.147 g

Triton X100.....1 ml

distilled water.....to 100 ml

- yields 0.5 M Tris, 1.5 M NaCl, and 10 mM salts

- use for making working buffer
- salts were added to the buffer to enhance binding of lectins to substrate
- detergent was added to prevent non-specific hydrophobic binding of lectins to substrate and non-substrate

16. Tris Working Buffer

Tris salt buffer .....10 ml

distilled water.....90 ml

- adjust to pH 7.2 with NaOH
- yields 0.05 M Tris, 0.15 M NaCl, and 1.0 mM salts
- use for preparing lectin, staining, and washing solutions

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Humason, G. L. 1972. Animal Tissue Techniques. W. H. Freeman & Co. San Francisco.

Mowry, R. W. 1963. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue 8GX and their combinations with the periodic acid-Schiff's reaction. Ann. N.Y. Acad. Sci. **106**: 402-423.

Spicer, S. S., Neubecker, R. D., Warren, L. and Henson, J. G. 1962. Epithelial mucins in lesions of the human breast. J. Nat. Cancer Inst. **29**: 963-975.

Appendix II HISTOLOGICAL METHODS

1. Alcian Blue pH 0.5 (Bancroft & Stevens 1990)

2 X xylene.....3 min. ea.

2 X 98% anhydrous ethanol.....3 min. ea.

95% ethanol.....3 min.

70% ethanol.....3 min.

50% ethanol.....3 min.

distilled water.....3 min.

0.2 N hydrochloric acid.....3 min.

alcian blue pH 0.5.....5 min.

blot dry, taking care not to touch sections

neutral red.....5 min.

distilled water.....3 min.

2 X anhydrous 98% ethanol.....5 sec. ea.

2 X xylene.....3 min. ea.

mount in DPX

RESULTS:

- strongly-sulphated glycoconjugates.....blue
- nuclei.....red

2. Alcian Blue pH 2.5 (Bancroft & Stevens 1990)

2 X xylene.....3 min. ea.

2 X 98% anhydrous ethanol.....3 min. ea.

95% ethanol.....3 min.

70% ethanol.....3 min.

50% ethanol.....3 min.

distilled water.....3 min.

3% acetic acid.....3 min.

alcian blue pH 0.5.....5 min.

3% acetic acid.....3 min.

neutral red.....5 min.

distilled water.....3 min.

2 X anhydrous 98% ethanol.....5 sec. ea.

2 X xylene.....3 min. ea.

mount in DPX

RESULTS:

- weakly-sulphated glycoconjugates.....blue
- nuclei.....red

3. Alcian Blue pH 3.2 (Bancroft & Stevens 1990)

2 X xylene.....3 min. ea.

2 X 98% anhydrous ethanol.....3 min. ea.

95% ethanol.....3 min.

70% ethanol.....3 min.

50% ethanol.....3 min.

distilled water.....3 min.

0.5% acetic acid.....3 min.

alcian blue pH 0.5.....5 min.

0.5% acetic acid.....3 min.

neutral red.....5 min.

distilled water.....3 min.

2 X anhydrous 98% ethanol.....5 sec. ea.

2 X xylene.....3 min. ea.

mount in DPX

RESULTS:

- carboxylated glycoconjugates .....blue
- nuclei .....red

4. Alcian Blue-Aldehyde Fuchsin (Spicer et al. 1962)

2 X xylene.....3 min. ea.
2 X 98% anhydrous ethanol .....3 min. ea.
95% ethanol.....3 min.
70% ethanol.....3 min.
50% ethanol.....3 min.
distilled water.....3 min.
aldehyde fuchsin.....20 min.
70% ethanol.....rinse
distilled water.....3 min.
3% acetic acid .....3 min.
alcian blue pH 2.5.....5 min.
3% acetic acid .....3 min.
distilled water.....3 min.
70 % ethanol.....3 min.
95% ethanol.....3 min.
2 X anhydrous 98% ethanol .....3 min. ea.
2 X xylene.....3 min. ea.
mount in DPX

RESULTS:

- sulphated glycoconjugates.....purple
- carboxylated glycoconjugates .....blue

5. Alcian Blue-Periodic Acid-Schiff's Reagent (Mowry 1963)

2 X xylene.....3 min. ea.
2 X 98% anhydrous ethanol .....3 min. ea.
95% ethanol.....3 min.
70% ethanol.....3 min.
50% ethanol.....3 min.
distilled water.....3 min.
3% acetic acid .....3 min.
alcian blue pH 2.5.....5 min.
3% acetic acid .....3 min.
running water.....3 min.
1% periodic acid .....10 min.
running water.....3 min.
Schiff's reagent .....15 min.
running water.....3 min.
3 X 0.5% sodium bisulphite .....1 min. ea.
running water.....5 min.
70% ethanol.....3 min.
95% ethanol.....3 min.
2 X anhydrous 98% ethanol .....3 min. ea.
2 X xylene.....3 min. ea.
mount in DPX

RESULTS:

- acid glycoconjugates.....blue
- neutral glycoconjugates.....magenta
- mixture of acid & neutral glycoconjugates.....blue-purple - mauve

6. Lectins

2 X xylene.....3 min. ea.

2 X 98% ethanol.....3 min. ea.

95% ethanol.....3 min.

70% ethanol.....3 min.

50% ethanol.....3 min.

distilled water.....3 min.

3 X Tris buffer.....3 min. ea.

Lectin.....20 min.

3 X Tris buffer with agitation followed by jet wash.....10 min. ea.

mount in non-bleach mountant

RESULTS:

Appendix II Table 1. Lectin Affinities

Lectin	Sugar Affinity	Flourophore
WGA	N-acetyl-D-Glucosamine	fluorescein
BS I	D-Galactose, N-acetyl-α-D-Galactosamine	rhodamine
SJA	N-acetyl-β-D-Galactosamine	fluorescein
PSA	α-D-Mannose	rhodamine
UEA I	α-L-Fucose	fluorescein

CONTROLS:

Negative controls consisted of the above protocol followed with lectin solutions incubated in a 10 mg/ml solution of appropriate sugar substrate for 60 minutes prior to use (Table 2). Positive controls included sections of human colon containing erythrocytes and blood vessel endothelium.

Appendix II Table 2. Negative Controls of Lectins

Lectin	Sugar Affinity	Sugar Used
WGA	N-acetyl-D-Glucosamine	N-acetyl-D-Glucosamine
BS I	D-Galactose, N-acetyl-α-D-Galactosamine	D(+)-Galactose
SJA	N-acetyl-β-D-Galactosamine	N-acetyl-D-Galactosamine
PSA	α-D-Mannose	D(+)-Mannose
UEA I	α-L-Fucose	α-L(-)-Fucose

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Bancroft, J. D., and Stevens, A. 1990. Theory and Practice of Histological Techniques. Churchill Livingstone. Edinburgh.

Mowry, R. W. 1963. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue 8GX and their combinations with the periodic acid-Schiff's reaction. Ann. N.Y. Acad. Sci. **106**: 402-423.

Spicer, S. S., Neubecker, R. D., Warren, L., and Henson, J. G. 1962. Epithelial mucins in lesions of the human breast. J. Nat. Cancer Inst. **29**: 963-975.

VITA

I was born September 21, 1961 in Calgary, Alberta. Most of my youth was spent in Edmonton, Alberta. I received a B. Sc. degree with specialization in Entomology from the University of Alberta in 1985. While at the U. of A. I had the distinct good fortune and pleasure to work for three years as a laboratory technician for Dr. M. S. Goettel during his tenure there as a Ph. D. candidate. It was in Mark's lab that I met my future wife, Constance Eileen Prockiw.

I received a M. Sc. in Entomology from the University of Guelph in 1988. While there I learned why westerners loathe people from the east.

My passions in life include my companion Connie, learning, motorcycles, single malt scotch, and the endless pursuit of a newer and faster computer.